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TITLE: Efficient and Rapid Development of Transgenic Hamster Models of TSEs Using a Radical New Technology

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<b>14. ABSTRACT</b> The development of blood-based or other diagnostic tests for the TSE diseases should be greatly facilitated by an integrated set of compatible resources: 1) a susceptible large animal (sheep) to provide blood or other substrates, 2) susceptible transgenic rodents that can be used as a sensitive bioassay, 3) standard reference samples of brain, blood, and plasma of sufficient volume to be used by the TSE community. We are breeding a flock of 50 genotyped sheep to select for the high scrapie susceptibility genotype VVRRQQ. All three lines of the mouse transgenics carrying sheep, human, and elk PrP have been now re-derived. We have observed the first transmission of the disease from our standard scrapie-infected sheep brain inoculum to the transgenic mice with sheep PrP and have completed an end-point dilution titration. We have pooled and aliquoted the infected sheep whole blood, and separately, plasma (100 liters total). The pooled whole blood and plasma have been inoculated into transgenic mice for limiting dilution titrations that will quantify their exact level of infectivity, thus increasing their value to researchers. A protocol for sharing or sale of these resources has been prepared and samples have already been distributed.					
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## INTRODUCTION

Scrapie infected sheep are the only practicable source of realistic quantities of blood for the efficient development and validation of tests and devices for the management of transfusion risks from TSEs in humans and our domestic animals. To meet this need, a standardized reference material and development resource for blood-based TSE diagnostics was created from blood of scrapie infected sheep. A flock of ~250 sheep genetically selected for high susceptibility to scrapie and indigenously infected with field scrapie strains naturally acquired in agricultural settings or from within the flock was supported by subcontract. During the terminal stage of disease, scrapie affected animals were euthanized by exsanguination and the blood separated into components and stored frozen. Approximately, 100 liters of plasma was later pooled and realiquoted into 1 liter or smaller portions for use as a consistent, homogeneous, standard source of blood infectivity. A doubly transgenic mouse, tg<sub>shp</sub>, overexpressing the sheep prion gene on a mouse PrP knockout background was used to measure the concentration of scrapie infectivity in this pool and a whole blood pool prepared in parallel. Titration was by the limiting dilution method developed by our laboratory for quantitation of TSE infectivity in low titer materials such as blood and urine. Three hundred tg<sub>shp</sub> mice were inoculated with plasma and two hundred with whole blood. After 18 months there were no infections. Subsequent analysis showed some heterogeneity in the tg<sub>shp</sub> mouse genetics that weakened the result but could not however, account for the lack of infections. Other investigators, concurrently showed that both scrapie and BSE can be transmitted by transfusion of 50 mls or more of whole sheep blood back into sheep. These same-species transmissions in sheep have established that there is TSE infectivity in the blood of TSE infected sheep. Therefore, the lack of infections in the tg<sub>shp</sub> mice is most likely due to insufficient sensitivity of tg<sub>shp</sub> to cross-species infection from sheep to mouse. Other sheep transgenics may be more sensitive but there is no comparative data using a single reference material by which to rank them. To meet this need we prepared a sheep scrapie brain derived reference material and are currently negotiating access to other ovine transgenics to make such a comparison. We are also offering aliquots of the sheep plasma pool to any laboratory that will attempt a measurement on their premises. The current status of this project is that we have produced a resource of great value for its consistency and abundance and we and others are using it as a development resource with the expectation that it contains TSE infectivity. However, the material still lacks validation that it does in fact contain infectivity. Moreover, its value would be greatly increased if the level of infectivity could be quantified.

## BODY

*History:* This project originated as a proposal to apply a radical new transposon technology developed by TOSK Inc. to introduce the human, sheep, and other prion genes into golden Syrian hamsters and to use the new transgenic animals both to measure the concentration of blood-associated infectivity in naturally infected hosts compared to rodent models, and also as a source of TSE infected blood with the PrP phenotype of the



transgene donor for diagnostic development. Hamsters have the advantage of an 8 fold larger blood volume compared to mice ( a serious technical limitation of the mouse model for blood studies). If cross-species transmissions occurred in the transgenics, it would confirm the presence of infectivity in the blood of the naturally infected hosts. If concentrations were similar to those in rodents it would validate, extend, and generalize our prior observations on blood infectivity in rodents to relevant TSE-affected species, and provide a means of direct measurement of blood infectivity in those species. The transgenics would also be a consistent source of TSE infected blood with the correct species-specific PrP for diagnostic development. Unfortunately, TOSK suspended transposon-based transgenics services in 2004 without explanation. To date no hamster transgenic has been produced either by conventional or transposon based techniques. Once it became clear to us that the new technology was not going to produce a transgenic hamster model, we reverted to the fall back position that we had proposed in the original submission which was to base our transmission attempts on conventional mouse transgenics. After consultation with NPRP personnel, a revised statement of work was submitted and approved and this report as is based on that revision.

*Background:* Our laboratory developed an innovative method of TSE infectivity measurement, called limiting dilution titration, that was far more sensitive and precise than all earlier methods. Using this method we showed that the concentration of TSE infectivity in scrapie infected rodent blood during symptomatic disease was only 10 infectious doses (ID)/ml and even less during the lengthy preclinical incubation period. Detection of 10 ID/ml or less, is a challenging problem even for conventional viruses which elicit antibodies and contain PCR detectable nucleic acids. TSE diseases have neither. Moreover, in blood, the normal form of the prion protein is present at 10,000 to 100,000 greater concentration than infectious conformer(s). First generation blood tests, or at least the proof of principle for such tests, might require an entire unit or more of blood to obtain sufficient PrP<sup>res</sup> mass for detection. TSE infected human blood is far too scarce, too valuable and too heterogeneous for use in development applications. The study of rodent blood has been very productive, and our laboratory has produced unit quantities (500 mls) of hamster blood for specific experiments, but it was costly and would not be practical for wide scale use. We estimate that production of unit quantities of blood from mice would be at least 10 times more costly than from hamsters. A source of TSE infected blood that could be obtained on the human scale would greatly facilitate the development, validation and comparison of new diagnostics and devices and any other strategy to manage the risks from TSE infected human blood. The only practicable source of TSE infected blood in the quantities required is from sheep.

Based on our experience with rodent models, the key elements required for a useful sheep model are: 1. A source of TSE infected sheep, preferably naturally infected. 2. A sensitive and specific monoclonal antibody against sheep prion protein. 3. A transgenic mouse sufficiently sensitive to sheep TSE infectivity to be infectable at the low level of TSE infectivity expected in blood. To obtain quantitative measurements of the concentration of TSE infectivity in sheep blood, the transgenic mouse would have to be as sensitive to infection by sheep scrapie as is a sheep. There could be no remaining species barrier to infection. It is not known whether any PrP transgenic, for any species,



yet meets this criteria. A stringent test for this level of sensitivity would be detection of infectivity in blood or plasma of the transgene donor in a limiting dilution titration.

*Preliminary results* In the late 1990's we established a collaboration with Dr. Marie Bulgin at the University of Idaho, Caine Veterinary Center in Caldwell, Idaho, who supplied us with scrapie infected sheep blood and other tissues on a small scale. From 2000 through 2005 we were awarded an NIH contract for diagnostics development that included funds for development of a sheep source of TSE infected blood. We had by then already recognized the considerable heterogeneity between the blood and plasma collected from individual sheep (and humans) and its confounding effects on the interpretability of diagnostic assay development. We needed a consistent source of blood and plasma. Whole blood proved impractical because it can not be stored as such. We decided to establish a plasma pool that was large enough that it could supply an extensive development effort at a scale of 100 ml aliquots. Through a subcontract with the Caine Veterinary Center, animals with terminal scrapie were euthanized by exsanguination and the blood express mailed to our laboratory where it was characterized and then separated into components and stored frozen. Under a subcontract to the Institute for Basic Research in New York we funded the development of antisheep PrP monoclonal antibodies resulting in several excellent antibodies including 4B4 and 7D9. Our subcontract with the Institute for Basic Research also supported the development of a conventional transgenic mouse carrying the sheep PrP gene on a mouse PrP gene knock out background, i.e. the tg<sub>shp</sub> mice contained multiple copies of the ovine PrP gene and no copies of the mouse PrP gene.

In the subsequent section we will report on the progress made on each element in the statement of work.

*Revised item of work 1. Support the scrapie infected flock at the Caine Veterinary Center at a sustainable, if reduced, number of animals consistent with the funding. We would recommend halving the total number of animals to fifty. Support the continued genetic monitoring of Dr. Detwiler's control flock.*

NPRP funding supported a subgroup of 50 animals from the scrapie infected Caine Veterinary Center sheep flock of 250 or so animals. It was uncertain whether a flock of only 50 animals would sustain the same high rate of natural infection as the larger flock and other sources of funding were found for the remaining animals. BREF was offered blood and other tissues from most of the sheep succumbing to scrapie. Sheep were originally bred for the scrapie susceptible ARQ genotype that is most common in North America. However, subsequent to the discovery in this flock of an even greater susceptibility of the VRQ genotype to oral infection, the likely route of natural infection, the breeding has been redirected to enrich for codon 136 V. (Table 1) (Publication 1) The sheep are at high risk of developing scrapie from natural exposure within the flock and virtually all ARQ and VRQ animals eventually develop scrapie. The flock produced 15 to 20 cases of naturally acquired sheep scrapie per year. Scrapie diagnosis was confirmed by clinical symptoms and IHC test (Table 2). These animals supplied the



tissues given in Table 3. Some of the genetics were conducted by Dr. Jurgen Richt, USDA, ARS, while the balance was funded from other sources.

Table 1. Genetic composition of BREF flock

years	genotype	Percent
9/1/05 - 8/31/06	AARRQQ	34.0
	AVRRQQ	44.6
	VVRRQQ	10.2
	other	11.3
9/1/06 - 8/31/07	AARRQQ	29.5
	AVRRQQ	48.2
	VVRRQQ	14.4
	other	7.9

Table 2. Clinical cases of scrapie in Caine Veterinary Center flock

	Entire flock		BREF animals	
year	natural infections	experimental infections	natural infections	experimental infections
2005-2006	21	14	20	7
2006-2007	17	27	15	13

Table 3. Infected sheep tissues 9/2005 to 9/2007

	All investigators	BREF
Tissue		
Blood, mls	129,290	65,270
Urine, mls	4,520	4,520
Tissue, gms	1488.5	65

*Revised item of work 2. Pool, and bring to homogeneity the sheep plasma and separately the frozen whole blood that we have collected over the last five years and redistribute each into aliquots that will serve as a common TSE infected blood reference material.*

Over 200 liters of blood was collected by exsanguinations of terminally affected animals over the course of this project. Each collection was characterized for cellular composition and hemoglobin on a Hemovet five part differential analyzer calibrated for sheep blood. It was then separated into components by centrifugation and the components either used immediately in assay development or stored at  $\leq 80^{\circ}\text{C}$  until later use. In 2006 over 100 liters of accumulated plasma was used to create three separate pools to serve as standard reference materials. Plasmas collected from naturally infected animals were pooled according to hemoglobin levels. The larger pool contained plasmas with very low levels of hemoglobin and a smaller pool contained plasmas with noticeably



visible hemoglobin. A third pool was created from plasmas collected from animals that had been experimentally inoculated with scrapie by the oral route.

Each plasma was thawed under controlled conditions specified by the American Red Cross for preparing human plasma pools. All plasma aliquots collected from a single animal were then pooled individually and analyzed for hemoglobin content spectrophotometrically as a measure of hemolysis. Three 50 ml aliquots of each individual plasma were then removed and stored separately at  $\leq 80^{\circ}\text{C}$ . The individual plasmas were then pooled in a large vessel, gently stirred for  $\geq 5$  minutes before redistribution into aliquots of 100, 500 and 1000 ml and refreezing at  $\leq 80^{\circ}\text{C}$ . Some of the plasmas from naturally infected animals contained much more hemoglobin than most. These were segregated into a separate pool on the basis of their hemoglobin content as assayed spectrophotometrically. Plasmas with  $A_{575} < 0.1$  (116 mg/L hemoglobin) were pooled in the low hemoglobin group and those with  $A_{575} \geq 0.1$  were pooled in the higher hemoglobin group. However, it is important to note that the acceptable range for hemoglobin contamination of plasma is 191 mg/L to 650 mg/L equivalent to  $A_{575} = 0.16$  to 0.56. None of the plasma pooled from naturally infected animals had an absorbance  $A_{575} > 0.2$  equivalent to 232 mg/L even in the higher hemoglobin pool. All of the individual plasmas collected from the experimentally inoculated group contained hemoglobin levels less than those giving absorbance  $A_{575}$  values  $< 0.2$  from the higher hemoglobin group of the naturally infected animals. They were therefore pooled as one group. Table 4 summarizes the final composition of the three pools.

Table 4. Plasma pools prepared from blood of scrapie infected sheep

<b>Pool</b>	<b>Batch 101 Grade A</b>	<b>Batch 102 Grade B</b>	<b>Batch 103</b>
Infectivity	Natural	Natural	Experimental
# individual plasmas / pool*	42	8	26
Hb $A_{575}\text{nm}$	0.047	0.122	0.046
Hb mg/L	55	144	54
# bottles 800 ml/btl	68 repository 2 VAMHCS	16 repository 2 VAMHCS (+1 x 300 ml at VAMHCS)	40 repository 1 VAMHCS
Total volume*	~ 56 L	~ 14.7 L	~ 32.8 L

\* Three 50 ml aliquots of the plasma from each individual were stored separately before pooling. This is equivalent to 11 L of additional plasma besides that given in the table.

Also, aliquots of 50 to 150 mls of whole blood were removed from the blood received from each individual animal prior to component separation and stored at  $\leq 80^{\circ}\text{C}$ . Thus in addition to the three pools summarized in table 4, we also have panels of whole blood and plasma collected from 76 sheep in the terminal stages of clinical scrapie infection.

There is no equivalent resource.



*Revised item of work 3. Develop a protocol for distribution of sheep resource materials to academic requesters and a price list for distribution of the same materials to commercial interests.*

All sheep materials that we have produced for our own use are also available to others. This includes stocks of scrapie infected blood, plasma, or brain from individual animals contributing to the production of the reference material pools described above as well as aliquots of the materials in those pools. We especially encourage the use of the pools for any work that will be used to compare one result with another whether in an individual laboratory or between laboratories and tests. The terms for supply differ for publicly-funded academic investigators and commercial clients. In both instances, however, sheep scrapie infected material can only be provided to organizations, whether academic or commercial, who provide copies of current USDA permits to acquire and use such material.

Academic investigators:

Aliquots are supplied to academic investigators for the cost of hazardous material shipping.

Commercial interests:

Aliquots of the sheep plasma reference material pools and sheep brain homogenate standards that we have developed are provided to commercial diagnostic developers or any other commercial organization according to the price list below. The commercial sale of these materials are conducted through the Baltimore Research and Education Foundation (BREF), the not for profit corporation of the VA Medical Research Service that administered the NIH and DoD funding for this project. The proceeds are used to support the storage, maintenance, ongoing characterization (including bioassay) and replenishment of these materials.

Price list for sheep scrapie infected reference materials:

**SSP-NPA**

Scrapie infected sheep plasma. Collected by exsanguination of sheep terminally ill with naturally acquired North American scrapie. Grade A      \$200 + \$10/ml

**SSP-NPB**

Scrapie infected sheep plasma. Collected by exsanguination of sheep terminally ill with naturally acquired North American scrapie. Grade B      \$200 + \$5/ml

**SSP-EP**

Scrapie infected sheep plasma. Collected by exsanguination of sheep terminally ill with North American scrapie acquired by experimental inoculation by the oral route.      \$200 + \$10/ml



**SBH-SHP**

Pooled brain tissue from sheep terminally ill with naturally acquired North American scrapie homogenized in phosphate-buffered saline. \$300/ml  
This stock had an infectivity titer of  $1 \times 10^7$  ID<sub>50</sub>/ml, when assayed by end point dilution titration in tg<sub>shp</sub> mice.

Price list for materials from individual animals:

**SSP**

Scrapie sheep plasma from an individual animal \$200 + \$5/ml

**SBH-SHI**

Brain tissue from a individual sheep terminally ill with naturally acquired North American scrapie homogenized in phosphate-buffered saline. (limited quantities available) \$250/ml

A final report has been prepared giving the history, methods, and characteristics of these materials which will serve as a draft manuscript for a publication that will more widely publicize these materials. However, we have been deferring publication until we have proof that these materials are actually infectious. As discussed in item 4 following, that proof has so far eluded us.

*Revised item of work 4. Use the transgenic mice carrying the sheep PrP gene that we have developed, to measure the infectivity in the sheep plasma pool and the whole blood pool using limiting dilution titration. This will accomplish one of the ultimate goals of the original proposal.*

Transgenic mice, tg<sub>shp</sub>, carrying the sheep PrP gene on a mouse PrP gene knock out background were previous developed for our use by subcontract to the Institute for Basic Research in Staten Island, NY. These mice have multiple copies of the sheep PrP gene with ARQ genotype and no mouse PrP gene. They were bred to homozygosity at the IBR and then rederived at Harlan Labs to rid them of several pathogens present in the IBR colonies that would be incompatible with the VA/U Maryland rodent colonies. We obtained the mice from Harlan. Their properties had not been characterized.

Our principle interest in these mice was as a means of :

1. Establishing for sure whether or not the blood and plasma of scrapie infected sheep contained TSE infectivity, and
2. Measuring by bioassay the concentration of the infectivity in the sheep plasma and brain pools that we had prepared.



There is a “species barrier” that typically reduces the efficiency of transmission of TSE infections between species by at least 1000 fold. This barrier is significantly lowered in transgenic mice bearing the transgene of the infecting species. This is readily seen as a lowering of the incubation time for transmissions from high titered brain homogenates. However, it is not known whether the barrier is eliminated entirely. To successfully demonstrate the presence of infectivity in the blood of a transgene donor or to measure the concentration of TSE infectivity in the blood of the transgene host, the barrier would have to be almost completely eliminated if the blood and plasma of transgene donor has a TSE infectivity concentration similar to that found in hamsters and mice, ~ 10 ID/ml. The reason that our laboratory has been so successful in quantifying the titer of the infectivity in the blood and urine of hamsters and mice is that the measurements have been made in the same species as the collected blood or urine. There is no species barrier. Even so we had to develop a novel method of detection with much greater sensitivity and precision than the end point dilution assay which has previously been the most sensitive and precise assay for TSE infectivity but insufficiently sensitive to measure the concentration of TSE infectivity in blood.

Therefore, as a first step towards meeting the goals of this section we attempted to evaluate the sensitivity of tg<sub>shp</sub> to sheep scrapie by conducting an end point dilution titration of the **SBH-SHP** scrapie infected sheep brain pool that we had prepared as a standardized material for our own use. The infectivity titer was  $1 \times 10^7$  ID<sub>50</sub>/ml, after a relatively short incubation time of six to eight months. We have no same species, sheep to sheep, titration with which to compare this value. However, in the few instances where sheep to sheep transmission have been conducted, sheep brain the titers were comparable - at most  $10^8$  ID<sub>50</sub>/ml with large standard errors. This result was sufficiently encouraging that we have undertaken a limiting dilution titration of scrapie infected sheep whole blood and the **SSP-NPA** plasma pool. Typically, we assay a total of 5 mls of blood or plasma to obtain a limiting dilution titer. The blood or plasma is inoculated intracranially, ic, 25 µl per mouse requiring 200 mice in all. To compensate for the potential reduced efficiency of a cross species transmission ~300 mice were inoculated intracranially with plasma and over 200 mice with whole blood, ~12.5 ml and over 500 mice total. There were no signs of clinically infected mice in nearly 600 days of observation.

All mice in this study were euthanized and their brains collected for analysis by Western blot for evidence of infection - specific forms PrP. This analysis is still ongoing (over 500 brains) however, there has been no detection of PrP<sup>res</sup> forms to date. However, we have discovered evidence of heterozygosity in at least some of the breeders contributing to this cohort of mice. This suggests genetic instability in this transgenic line. As this is a new strain of transgenic that has only been through a few cycles of breeding, this is possible. This greatly complicates the analysis of this data. Since we have tissue from each mouse in the study we will be able to sort out the zygosity of each mouse. Since we do not have a titration for heterozygous mice we do not know the relative sensitivity of heterozygotes to infection. The effect will be to reduce the total number of homozygous tg mice that are in the study and perhaps the sensitivity of the analysis to detect sheep infectivity. Nevertheless, in spite of these difficulties, some analysis is possible. We can

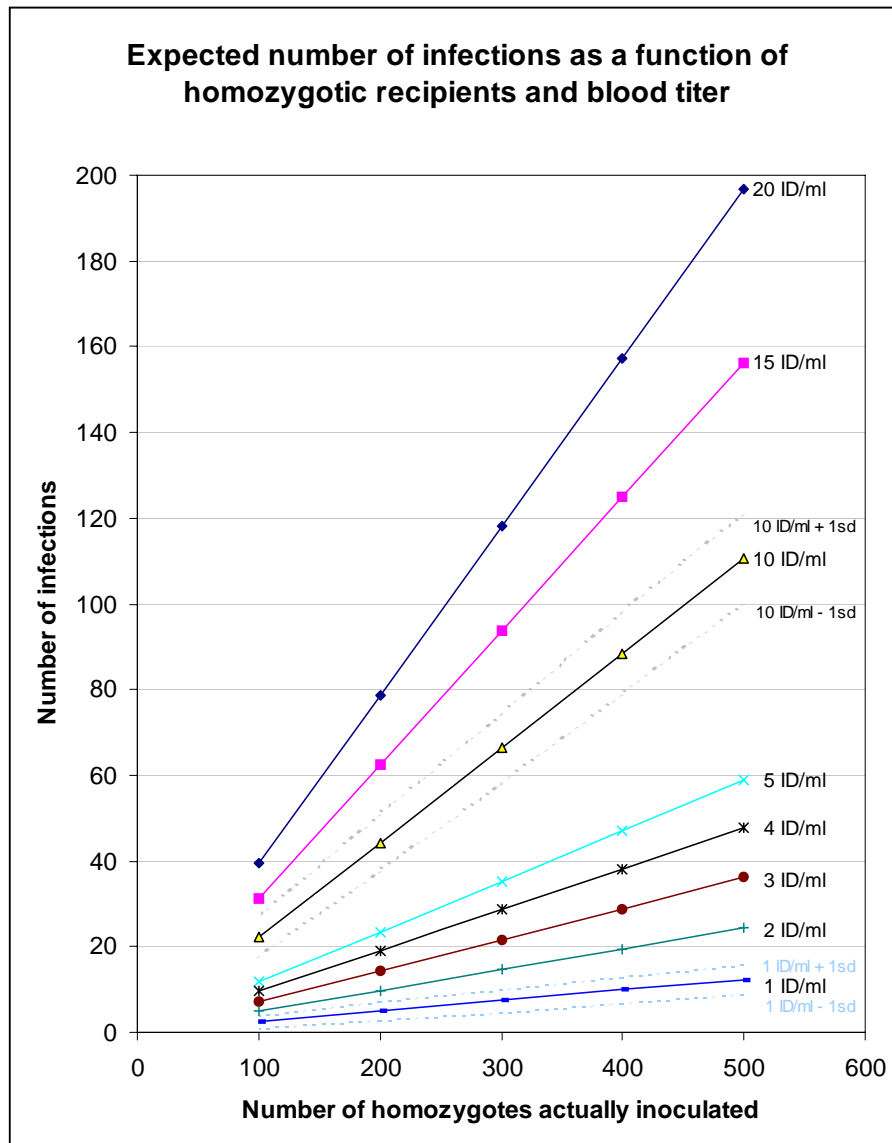


calculate that had the titer of TSE infectivity in sheep blood and plasma been 10 ID/ml we still would have expected  $22 \pm 5$  infections even if only 100 of the 500 animals inoculated were fully as sensitive as the homozygotes. This value is  $12 \pm 3$  for 5 ID/ml and still a detectable 2 infections at 1 ID/ml. These calculations are summarized in Table 6 and Figure 1.

**Expected number of infections as a function of homozygotic recipients and blood titer**

Total volume, ml	12.5ml		inoculum, ml 0.03													
Titer, ID/ml	20		15		10		5		4		3		2		1	
$m = \text{ID}/\text{inoculum}$	0.5		0.375		0.25		0.125		0.1		0.075		0.05		0.025	
$P(0) = e^{-m}$	0.607		0.687		0.779		0.882		0.905		0.928		0.951		0.975	
$1 - P(0)$	0.393		0.313		0.221		0.118		0.095		0.072		0.049		0.025	
homozygotes	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$	Infections	$\pm \text{sd}$
500	197	14	156	13	111	11	59	8	48	7	36	6	24	5	12	4
400	157	13	125	11	88	9	47	7	38	6	29	5	20	4	10	3
300	118	11	94	10	66	8	35	6	29	5	22	5	15	4	7	3
200	79	9	63	8	44	7	24	5	19	4	14	4	10	3	5	2
100	39	6	31	6	22	5	12	3	10	3	7	3	5	2	2	2

Figure 1.





The lack of infections could indicate that there is no sheep scrapie infectivity in either the whole blood or plasma samples. However, it is more likely that the transgenic has failed this very stringent test of sensitivity. This is especially true now that others have repeatedly shown that scrapie and BSE are both transmissible in sheep via transfusion of whole blood. Admittedly the volumes transfused are ten to twenty times greater than those inoculated in this experiment. However, transfusion transmission appears to be quite efficient in sheep suggesting that the titer is at least comparable to that found in rodents. On the basis of this evidence we have concluded that the tg<sub>shp</sub> line is not sufficiently sensitive to make this measurement and we do not intend to pursue further development of this particular sheep transgenic mouse line.

In the mean time several alternative ovine PrP transgenics have been developed by other laboratories. One or more of these transgenics may be more sensitive than tg<sub>shp</sub> but there is not, as yet, any comparative data using a single reference material by which to rank them. It was in part to meet this type of need that we prepared a sheep scrapie brain-derived reference material. We are currently negotiating access to other ovine transgenics to make such a comparison. We are also offering aliquots of the sheep plasma pool and the brain reference material to laboratories that do not wish to donate their tg mouse lines but would be willing at least to make a comparative measurement on their own premises.

The current status of this project is that we have produced a resource of great value for its consistency and abundance and we and others are using it as a development resource with the expectation that it contains TSE infectivity. However, the material still lacks validation that it does in fact contain infectivity. Moreover, its value would be greatly increased if the level of infectivity could be quantified.

*Revised item of work 5. Continue to develop the short incubation time model of sheep scrapie by breeding for VVQQ genotype and infecting with inocula prepared from the first passage transmissions.*

The sheep scrapie model would also be greatly improved if the incubation time of the disease could be reduced. The brain-derived reference material that was prepared as part of this project unexpectedly produced a dramatically shorter incubation time in some animals even when inoculated orally. Scrapie seldom has less than a two year incubation time in sheep and more typically takes four years or more to cause disease. This inoculum produced clinical disease in less than one year in several animals. This is comparable to rodent incubation times. It turned out that the short incubations were concentrated in animals with AVRRQQ or VVRRQQ genotypes. There may have been some synergy between the inoculum and the genotype as the VRQ genotype is also represented in the inoculum. To test these possibilities with the objective of developing an even shorter incubation time model in sheep, we needed to increase the representation of the VRQ genotype in the flock and also obtain more tissue from VRQ infected animals from which to prepare inoculum.

This was facilitated by the identification of a ram in the high susceptibility flock with a VVRRQQ genotype. This ram was used to increase the production of VVRRQQ animals. Since he was a member of the high incidence flock and had the highly susceptible genotype himself, he has also succumbed to the disease but not before producing another 5 VVRRQQ lambs in 2006 and 12 VVRRQQ in 2007. The five 2006 VV animals are still alive, but



clinical. The 12 from 2007 are also still alive. All 6 of the 2005 VV cohorts to this ram died of scrapie between the ages of 9 and 18 months. The discovery of this highly susceptible and short incubation time genotype in sheep has made this model even more advantageous as an economical source of TSE infected blood, urine and other tissues for development, testing and validation purposes.

#### **KEY RESEARCH ACCOMPLISHMENTS:**

- Sheep brain reference materials: We have pooled sheep brains that have been carefully selected for giving strong Western blot signals for PrP<sup>res</sup> to produce a highly infectious brain reference material for development of TSE management strategies and for comparison and validation of experimental results, transgenic sensitivities, diagnostics, and other assays based on sheep scrapie infectivity.
- Sheep blood and plasma reference materials: We have collected, characterized and pooled over 100 liters of high grade sheep plasma and whole blood collected from scrapie infected sheep in terminal disease for use in the development and validation of diagnostics and other strategies for managing the risks from blood-borne TSE infectivity.
- We have discovered a very short incubation time model of scrapie in sheep and are developing it into a research tool. (publication 1).
- We developed and characterized a transgenic mouse carrying the sheep PrP gene on a mouse PrP knockout that shows high efficiency of cross-species transmission of scrapie from sheep brain to mouse even though this efficiency was not great enough to detect infectivity in sheep blood.

#### **REPORTABLE OUTCOMES:**

To date there have been two publications from the Caine Veterinary Center that have been supported by this funding (listed below). A report on the production and characterization of the plasma reference material has been written which will serve as a draft for a publication presuming that we can demonstrate infectivity in this material. A collaborative study with Dr. Juergen Richt is investigating the activity of the sheep brain reference material in various genotypes of sheep and transgenic mice. This will also be published upon completion of the work which may require several more years. We will also publish a characterization of the tg<sub>shp</sub> transgenic with Dr. Rubenstein who constructed the mouse. Even though this mouse does not meet our requirements for sensitivity, it has shown remarkable sensitivity to cross species transmissions from sheep brain, and may rank highly in comparison with other ovinized transgenics. This publication will also highlight the scrapie infected sheep brain reference material that we created to make this measurement and its availability for comparative measurements by others.

#### **CONCLUSIONS:**

We have produced a variety of scrapie infected reference materials from brain and blood of sheep naturally infected with scrapie for use in the development and validation of TSE



diagnostics and other TSE management efforts. We have also developed potent anti-sheep PrP monoclonal antibodies, and a PrP ovinized transgenic mouse that is highly susceptible to cross-species infection from sheep. We have discovered a sheep genotype with high sensitivity to natural infection and an incubation time comparable to that for TSE infections of rodents. Together these elements satisfy all of the requirements for an effective sheep scrapie resource. There is still a need for a transgenic mouse of sufficient sensitivity for efficient infection by scrapie infected sheep blood.

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Bulgin MS, Sorensen SJ, Matlock ME. Association between incubation time and genotype in sheep experimentally inoculated with scrapie-positive brain homogenate.. American Journal of Veterinary Research. 2006. 67: 498-504.

Bulgin MS, Sorensen Melson S. What veterinary practitioners should know about scrapie. J. Amer. Vet. Med. Assoc. 2007 230:1158-1164.

## **APPENDICES:**

### **Publications**

#### *Published*

Bulgin MS, Sorensen SJ, Matlock ME. Association between incubation time and genotype in sheep experimentally inoculated with scrapie-positive brain homogenate.. American Journal of Veterinary Research. 2006; 67: 498-504.

Bulgin MS, Sorensen Melson S. What veterinary practitioners should know about scrapie. J. Amer. Vet. Med. Assoc. 2007; 230:1158-1164.

Hamir AN, Kunkle RA, Bulgin MS, Rohwer RG, Gregori L, Richt JA. Experimental transmission of scrapie agent to susceptible sheep by intralingual or intracerebral inoculation. Can J Vet Res. 2008 January; 72:63-67.

#### *Submitted:*

Bulgin MS, Sorenson Melson, S, Matlock ME. A Comparative Study Utilizing Immunohistochemistry on Lymphoid Tissue of the Third Eyelid and Rectal Mucosa for the Diagnosis of Scrapie in Sheep.

### **Animal Use Approvals**

University of Idaho

Baltimore Research and Education Foundation, Inc.



# Association between incubation time and genotype in sheep experimentally inoculated with scrapie-positive brain homogenate

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**Objective**—To compare incubation time and clinical signs of scrapie in codon 136/171 alanine-valine/glutamine-glutamine (AVQQ) experimentally inoculated sheep with that in sheep with the more common 136/171 AAQQ genotype.

**Animals**—60 Suffolk sheep.

**Procedure**—Twenty-seven 171 QQ ewes purchased from 2 private flocks were bred with a 171 QQ Suffolk ram before being inoculated with a 20% solution of scrapie-positive brain homogenate (5 mL, PO) from sheep containing genotypes 136/154/171 AA/arginine-arginine (RR)/QQ, AVRRQQ, and VVRRQQ that had died of scrapie. Ewes had 33 lambs, which were inoculated in the same manner on the day of birth.

**Results**—All 16 genotype 136/154/171 AVRRQQ sheep that died of scrapie were 9 to 11 months of age; clinical signs lasted 1 day to 3 weeks with no wasting and only mild pruritus. The first AARRQQ sheep died with typical clinical signs of scrapie 27 months after inoculation, and 14 were still alive 37 to 42 months after inoculation. The 136/171 AVQQ sheep had minimal accumulation of modified cellular protein (PrP<sup>Sc</sup>) as determined by immunohistochemical (IHC) staining within affected cells; thus the severity of clinical signs and time of death were not associated with brain lesions or the amount of PrP<sup>Sc</sup> in brain tissue of 136/154/171 AVRRQQ sheep as determined by IHC staining.

**Conclusions and Clinical Relevance**—The rapid incubation time may have been influenced by the codon 136 genotype, a new unreported valine (V)-dependent strain of scrapie similar to strain SSBP/1, or the inoculum may have contained a traditional strain and a V-dependent or SSBP/1-like strain of scrapie. (*Am J Vet Res* 2006;67:498–504)

Scrapie is a progressive, debilitating neurologic illness of sheep and goats that is thought to always result in death. Presently, scrapie is believed to be the result of accumulations in the brain of posttranslational PrP<sup>Sc</sup>. Experimental infectivity studies<sup>1</sup> have established that the presence of PrP<sup>Sc</sup> is a reliable indicator of the presence of the infective agent. The PrP<sup>Sc</sup> is

believed to act as a template for the conversion of PrP<sup>C</sup> or prion protein to PrP<sup>Sc</sup> in genetically susceptible sheep.

Nucleotide variants in the PRNP may affect the translation of codons 136, 154, and 171, which are most commonly associated with susceptibility to the disease. Codon 136 codes for either V or A; codon 154 codes for R or H, and codon 171 codes for Q, K, H, or R. Resistance against scrapie appears to be increased by 136 A,<sup>2,3</sup> 154 H,<sup>4</sup> and 171 R.<sup>5</sup> In the United States, the disease affects mainly blackface breeds of sheep (Suffolk and Hampshire and crosses thereof), contributing approximately 96% to the disease prevalence.<sup>6</sup> Also in the United States, the disease is mainly associated with polymorphism at codon 171, as V at codon 136 and H at codon 154 are rare.<sup>7,8</sup> Homozygosity of RR at codon 171 appears to confer resistance; the presence of 1 R decreases the incidence of scrapie substantially, whereas homozygosity of glutamine (QQ) and presence of H (QH or HH) render sheep susceptible to the disease<sup>9</sup> and have been responsible for all infections of scrapie reported in the United States, except for a few cases of scrapie in sheep with codon 171QR.<sup>6,10</sup>

In the United States and Great Britain, efforts to control and perhaps eliminate the disease on the basis of selective breeding of sheep having at least 1 R at the 171 site and to remove 171 QQ sheep from flocks are in progress.<sup>10,11</sup> The expectation is that the remaining sheep will be resistant to scrapie infection. Little attention is given to the genetics at codon 136 in the United States because it is reported that most Suffolk and Hampshire sheep are 136 AA.<sup>7,8</sup>

There is 1 published report<sup>10</sup> of natural scrapie in known 136 AV Suffolk sheep in the United States. However, we report the findings when brain tissue

PrP <sup>Sc</sup>	Modified cellular protein
PrP <sup>C</sup>	Normal cellular protein
PRNP	Prion protein gene
V	Valine
A	Alanine
R	Arginine
H	Histidine
Q	Glutamine
K	Lysine
SBH	Sheep brain homogenate
TB	0.1M Tris buffer (pH, 7.5) containing 1M NaCl and 0.25% Tween 20
TBGS	TB containing 10% goat serum
ID	Identification
MT	Methionine/tryptophane
NPU	Neuropathogenesis unit

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from sheep testing positive for scrapie was inoculated into 136/154/171 AVRRQQ Suffolk sheep at birth and at 12 months of age. The purpose of the study reported here was to compare the incubation time and clinical signs of disease in sheep with the 136/171 AVQQ genotype with that in sheep with the more common 136/171 AAQQ genotype in this group of sheep.

## Materials and Methods

**Inoculum**—Brains from 7 clinically affected 171 QQ sheep testing positive for scrapie were verified to have PrP<sup>Sc</sup> by IHC and western blot testing of brain obex. Whole brains were singly homogenized, combined, and diluted to a 20% solution with PBS solution. The combined scrapie-positive SBH was frozen and stored at  $-70^{\circ}\text{C}$ . Samples of the single homogenates were later sequenced; 5 sheep were 136/154/171 AARRQQ, 1 was AVRRQQ, and 1 was VVR-RQQ.

**Sheep and treatment**—Twenty-seven Suffolk ewes, approximately 9 months old, with the genotype 171 QQ were purchased from 2 private owners in October 2001. Scrapie had never been observed in either flock and has not been observed since. In November 2001, ewes were exposed to a Suffolk ram with the genotype 136/154/171 AVRRQQ. In December 2001, sheep were given 5 mL of the thawed 20% SBH solution, which was deposited superficially on the back of the tongue via a dose syringe. Other than thawing, there was no special treatment of the inoculum. Thirty-three lambs were born during April 2002, and on the day of birth, each lamb was also given 5 mL of 20% SBH solution in the same manner.

Ewes and lambs were housed in an outside drylot pen with fence-line feeders and shelter from inclement weather. The study was approved by the University of Idaho Animal Care and Use Committee. Their base diet was alfalfa hay, and grain was fed during pregnancy and lactation. Heated, automatic waterers provided water ad libitum. A trace mineral salt was provided. Two technicians checked sheep twice daily and recorded any problems or abnormal behavior. Sick sheep with any illness were moved into a separate pen for specific care or supportive treatment as needed.

**Biopsy protocol**—A biopsy specimen was obtained from the lymphoid tissue of the third eyelid as described,<sup>12</sup> except xylazine (0.1 mg/kg) was given IV for sedation and anesthesia. For mandibular lymph node biopsies, the area beneath the base of the ear was prepared for surgery after xylazine injection. The lymph node was palpated just medial to the angle of the mandible, and an incision was made over the top of the lymph node with a disposable scalpel. After the lymph node was identified, it was grasped with a pair of forceps and removed with either scissors or scalpel. One or 2 sutures were placed in the skin, and sheep were permitted to recover. Instruments were soaked in 2.5N NaOH for at least 24 hours, rinsed, then sterilized in an autoclave for reuse.

**Necropsy protocol**—Ewes and lambs were euthanatized when they could no longer hold themselves in sternal recumbency, and necropsies were performed. Fresh samples of tissues in which gross abnormalities were detected on necropsy were taken for bacteriologic examination and placed into neutral-buffered 10% formalin for histologic examination. Several areas of the brain and spinal cord were also sectioned for H&E staining and microscopic evaluation. Samples of brain, palatine tonsils, retropharyngeal lymph nodes, mandibular lymph nodes, lymphoid tissue of the third eyelid (if still present), mesenteric lymph node, and ileum with Peyer's patches were obtained; 1 sample from

each tissue was placed into neutral-buffered 10% formalin for IHC, and a second sample was frozen at  $-20^{\circ}\text{C}$  for future experimental protocols.

**IHC testing**—Fixed, paraffin-embedded tissues were cut at 5  $\mu\text{m}$ , and resulting slides were processed by use of a microprobe system.<sup>a</sup> After tissues were deparaffinized and hydrated, the paired slides were placed in an autoclave at  $121^{\circ}\text{C}$  for 30 minutes in TB. The cooled slides were rinsed in TBGS used also to make a 1:1,000 dilution of the monoclonal antibody (cell line F89/160.1.5<sup>b</sup>), which was applied to the slides for 12 hours at  $2^{\circ}\text{C}$  incubation.

Slides were rinsed with TBGS, and the secondary antibody (biotinylated anti-mouse IgG, H and L,<sup>c</sup> heavy and light chains), diluted in a ratio of 1:200 in TBGS, was added for 30 minutes. After rinsing with TB, slides were loaded with avidin-alkaline phosphatase and incubated for 20 minutes. Slides were rinsed several times with 0.1M Tris HCl buffer (pH, 8.2) containing 0.25% Tween 20. Alkaline phosphatase substrate was prepared with 0.1M Tris HCl buffer (pH, 8.2) containing 0.25% Tween 20 and 0.1 mL of levamisole, then loaded onto slides. Slides were incubated for 5 minutes twice, then once for 10 minutes, rinsed with water containing 0.25% Tween 20, and counterstained with hematoxylin with TB used as a bluing reagent. Slides were dehydrated in graded alcohols and cleared, and a cover slip was placed on each slide.

For lymphoid tissue to be considered negative for scrapie, a minimum of 5 lymphoid follicles was required on the slide for evaluation. The presence of 4 negative follicles, for example, was considered a no test. This was only a problem in the case of eyelid tissue.

## Results

Two lambs died at 0.7 and 1.4 months of age. In those lambs, the cause of death was determined to be starvation and pneumonia. Tissues were not retained for scrapie testing. Two other lambs died at 4.6 and 5.7 months of age of pneumonia, and results of IHC tests were negative. Five months after inoculation, 1 ewe died from a prolapsed uterus; this ewe was not tested for scrapie. One ewe died 9 months after inoculation during anesthesia for herniorrhaphy; PrP<sup>Sc</sup> was not detected in tissues via IHC at the time of death (Table 1).

In January of 2003, 12 and 9 months after inoculation of ewes and lambs, respectively, biopsy specimens of third eyelid lymphoid tissue were obtained from all inoculated sheep and tested for PrP<sup>Sc</sup> via IHC. Only specimens from 2 adults and 1 lamb tested positive. As of June 2005, the lamb and one of those adult sheep were alive 36 and 42 months after inoculation, respectively. The other adult had clinical signs compatible with scrapie<sup>13</sup> for 3 weeks and was euthanatized 16 months after inoculation (in April 2003).

Twelve months after inoculation of adult sheep, 2 ewes, (ID No. 290 and 298) were ataxic and had difficulty maintaining their balance (Table 1). Five days later, one of those ewes (ID No. 290) was unable to maintain sternal recumbency and was euthanatized; all tissues, including the third eyelid, tested positive for PrP<sup>Sc</sup> (Table 2). The other ewe (ID No. 298) was euthanatized 3 weeks after showing clinical signs compatible with scrapie; all tissues, except the mandibular lymph node, tested positive for PrP<sup>Sc</sup>. One adult ewe (ID No. 283) in which tissue from the third eyelid tested positive was euthanatized 3 weeks after developing clinical signs of ataxia. Thirteen months after inocula-



Table 1—Summary of sheep with genotype 136/171 AVQQ dying after inoculation with a 20% solution of scrapie-positive brain homogenate (5 mL, PO) as an adult (12 months old) or as lambs at birth.

ID	Inoculation date	Cause of death	Incubation time (mo)	Clinical signs	Scrapie diagnosis
Inoculated as an adult					
283	12/19/01	Euthanatized	15.3	Mild pruritis, bunny hopping	Pos
290	12/19/01	Euthanatized	11.9	Left lateral recumbency	Pos
293	12/19/01	Euthanatized	13.3	Left lateral recumbency	Pos
298	12/19/01	Euthanatized	12.6	Left lateral recumbency	Pos
300	12/19/01	Uterine prolapse	5.4	None	No test
305	12/19/01	Died—anesthesia	7.3	None	No test
Inoculated at birth					
2101	3/21/02	Pneumonia	1.4	None	No test
2107	4/10/02	Euthanatized	10.0	Left lateral recumbency	Pos
2122	4/15/02	Euthanatized	11.0	Ataxic	Pos
2124	4/15/02	Euthanatized	9.6	Left lateral recumbency	Pos
2127	4/15/02	Euthanatized	11.0	Ataxic	Pos
2128	4/18/02	Euthanatized	11.1	Bunny hopping	Pos
2133	4/22/02	Found dead	0.7	None	No test
2140	4/23/02	Found dead	4.6	None	Neg
2142	4/20/02	Euthanatized	11.3	Bunny hopping	Pos
2144	4/22/02	Euthanatized	11.0	Bunny hopping, mild pruritis	Pos
2146	4/23/02	Euthanatized	8.8	None	Pos
2150	4/23/02	Euthanatized	9.3	Left lateral recumbency	Pos
2081	5/19/02	Euthanatized	9.4	Left lateral recumbency	Pos
2121	4/15/02	Euthanatized	9.2	Left lateral recumbency	Pos
2123	5/3/02	Euthanatized (pneumonia)	5.7	Pneumonia	Neg
2136	4/2/02	Euthanatized	10.8	Right lateral recumbency	Pos
For lymphoid tissue to be considered negative for scrapie, a minimum of 5 lymphoid follicles was required on the slide for evaluation; ≤ 4 follicles was considered a no test. Pos = Positive. Neg = Negative.					

tion, 1 ewe (ID No. 293) became recumbent and was euthanatized. This ewe had appeared clinically normal 1 day earlier. All tissues, except for lymphoid tissue of the third eyelid, tested positive for PrP<sup>Sc</sup> via IHC.

These 4 sheep were in good body condition and did not have the wasting commonly associated with naturally occurring scrapie; 3 sheep did not have pruritis and 1 sheep had minimal pruritis, and they did not have visible tremors commonly seen in sheep with classic signs of scrapie. Clinical signs in these sheep progressed from lack of coordination to being unable to stand in a brief period of time: approximately 3 weeks, sometimes within 18 hours. The genotype of these 4 ewes was 136/154/171 AVRRQQ.

In the first 3 months of 2003, 12 lambs, 36.4% of those born in 2002 and inoculated at birth, died of scrapie. All had an incubation time < 12 months, (mean, 10.2 months), and duration of clinical signs was < 2 weeks. In those lambs, brain and mesenteric lymph node tissue tested positive for PrP<sup>Sc</sup> via IHC. The genotype of those lambs was 136/154/171 AVR-

RQQ. Other tissue results from these lambs are described (Table 2).

The PrP<sup>Sc</sup> accumulation in tissues of SBH-inoculated AVRRQQ sheep was notably less as determined by IHC than that of sheep naturally infected, being fine granular deposits lightly sprinkled through affected cells and often difficult to see. Findings from several of the first cases of scrapie in sheep were shared with personnel in the USDA Agricultural Research Service<sup>d</sup> to substantiate the diagnosis. Results of all bacterial cultures of brain and spinal cord were negative for *Listeria* spp, and other infectious bacteria and histologic examinations of sections obtained from obex, cerebrum, cerebellum, and spinal cord did not reveal classic scrapie lesions.

Sequencing the PRNP gene from brain tissue of 13 AVRRQQ sheep that died with a decreased incubation time was performed,<sup>e</sup> starting at amino acid 54 through amino acid 254. The first 53 amino acids were not sequenced because there are no reported critical polymorphisms in these areas. There were 3 sheep that had an MT polymorphism at codon 112 (Table 3). This



Table 2—Immunohistochemistry test results for detection of PrP<sup>Sc</sup> performed on lymphoid and brain tissue from sheep inoculated with a 20% solution of scrapie-positive brain homogenate (5 mL, PO) as an adult (12 months old) or lambs at birth.

Sheep ID	Third eyelid lymphoid tissue*	Palatine tonsil	Mandibular lymph node	Retropharyngeal lymph node	Mesenteric lymph node	Brain obex
Inoculated as an adult						
283	Pos	Pos	Pos	Pos	Pos	Pos
290	Pos	Pos	Pos	Pos	Pos	Pos
293	Neg	Pos	Pos	Pos	Pos	Pos
298	Pos	Pos	Neg	Pos	Pos	Pos
305	Neg	Neg	Neg	Neg	Neg	Neg
Inoculated at birth						
2081	Neg	Neg	Neg	Pos	Pos	Weak Pos
2101	NS	NS	NS	NS	NS	
2107	Neg	Pos	Neg	Pos	Pos	Pos
2121	Neg	Neg	Neg	Neg	Neg	Weak Pos
2122	Neg	Pos	Pos	Pos	Pos	Pos
2123	Neg	Neg	Neg	Neg	Neg	Neg
2124	Neg	Neg	Pos	Pos	Pos	Pos
2127	Neg	Pos	Pos	Pos	Pos	Pos
2128	Neg	Pos	Pos	Pos	Pos	Pos
2136	Pos	Pos	Neg	Pos	Pos	Weak Pos
2140	Neg	NS	Neg	Neg	Neg	Neg
2142	Neg	Pos	Pos	Pos	Pos	Pos
2144	Neg	Pos	Pos	Pos	Pos	Pos
2146	Pos	Neg	Neg	Neg	Pos	Pos
2150	Neg	Neg	Neg	Neg	Pos	Pos

\*Positive third eyelid lymphoid tissue results are combined biopsy and necropsy results.  
 NS = No sample obtained.  
 See Table 1 for remainder of key.

Table 3—Results of prion protein sequencing of 13 inoculated 136/154/171 AVRRQQ sheep that died from scrapie with an incubation time ≤ 15.3 months.<sup>a</sup>

Sheep ID	Codon 136	Codon 154	Codon 171	Other polymorphisms
283	VA	RH	QQ	NA
290	VA	RR	QQ	NA
293	VA	RR	QQ	NA
298	VA	RR	QQ	NA
2107	VA	RR	QQ	NA
2122	VA	RR	QQ	NA
2124	VA	RR	QQ	NA
2127	VA	RR	QQ	Codon 112 MT
2128	VA	RR	QQ	Codon 112 MT
2142	VA	RR	QQ	Codon 112 MT
2144	VA	RR	QQ	NA
2146	VA	RR	QQ	NA
2150	VA	RR	QQ	NA

M = Methionine. T = Threonine. NA = Not applicable.

polymorphism has not been involved in resistance or susceptibility to scrapie. There were no additional polymorphisms or amino acid changes divergent from the known sheep PrP sequence.

The IHC results for third eyelid biopsy specimens from all dead lambs that were inoculated at birth were negative for PrP<sup>Sc</sup>, except for 1 lamb (ID No. 2146). The IHC results for third eyelid biopsy specimens in 1 other lamb (ID No. 2112) were positive also, but this lamb's genotype is 136/154/171 AARRQQ, and the lamb was still alive at the time of publication. The third eyelid tissue of 1 lamb (ID No. 2136) that originally tested negative for PrP<sup>Sc</sup> on biopsy tested positive for PrP<sup>Sc</sup> at the time of death.

All sheep (20 adults and 11 lambs) that were alive as of July 2004 (26 to 31 months after inoculation) were genotype 136/154/171 AARRQQ. The first scrapie-positive AAQQ sheep died 32 months (August 2004) postinoculation after having classic clinical signs of scrapie for several months. Fourteen of 31 AAQQ inoculated sheep and lambs were still alive 37 to 42 months, respectively (as of June 1, 2005), after inoculation.

## Discussion

The study reported here was initially undertaken to provide additional scrapie-positive tissues and blood for research. At that time, codon 136/154 genetics were



not considered particularly important in the United States, and only after inoculated sheep began to die at an early age was genotyping for the 136/154 codons performed. Because the 136 AV genotype was believed to be rare, the fact that many sheep in our study died from scrapie within 15 months and that they were all 136 AV was completely unexpected.

Six of 30 (20%) sheep (5 ewes and 1 ram) chosen from 2 private flocks on the basis of 171 QQ genotype were 136 AV, suggesting that the 136 AV genotype in some sheep flocks in the United States is not as rare as reported. The 136 V allele was also detected in our naturally infected research flock at a prevalence of 18.8%. In this flock, we found that the genotype also affected the incubation time, which was in agreement with results reported in sheep of all breeds in Great Britain.<sup>14</sup>

Typically, clinical signs in sheep with scrapie include wasting, incoordination and weakness of the hind limbs, pruritus, CNS signs, and behavioral changes that last 3 months or more.<sup>13</sup> In our study, duration of clinical signs in inoculated AVRRQQ adult sheep and lambs from the first observed clinical sign to complete inability to stand continued for as little as 1 day to less than a month. Wasting was not detected and pruritus was rare. A producer or veterinarian would not have diagnosed scrapie in sheep with these clinical signs. In most diagnostic laboratories, samples from young adult sheep that die suddenly and yield no diagnosis are never considered for IHC testing unless perhaps sheep were from a flock known to have scrapie.

Other remarkable disparities included differences in the accumulation of PrP<sup>Sc</sup> in lymphoid tissue. In our naturally affected sheep, PrP<sup>Sc</sup> has been detected in the lymphoid tissue of the third eyelids of 57% of sheep testing positive for PrP<sup>Sc</sup>,<sup>15</sup> whereas in this study, third eyelid lymphoid tissue from only 5 (31.2%) inoculated 136/154/171 AARRQQ sheep tested positive for PrP<sup>Sc</sup> at death. Additionally, in dead sheep inoculated at birth, palatine tonsils and mandibular and retropharyngeal lymph nodes had lower rates of IHC positive diagnoses (59%, 50%, and 75%, respectively) than mesenteric lymph nodes and brains (100%), whereas in naturally infected sheep, the mesenteric lymph nodes had the lowest percentage of IHC positive diagnoses (66%).<sup>15</sup> Perhaps the long incubation time permitted a longer duration for absorbed PrP<sup>Sc</sup> to migrate, replicate, and accumulate in the CNS and lymph nodes. Concentration of PrP<sup>Sc</sup> IHC staining in brains (obex) of all inoculated sheep was much less than in naturally infected sheep, requiring that additional time be spent searching slides for cells containing PrP<sup>Sc</sup>. Furthermore, no histologic lesions were detected in obex, cerebrum, cerebellum, and medulla oblongata; therefore, the cause of the rapid and severe clinical signs is not known. The amount of PrP<sup>Sc</sup> has been measured in scrapie-infected sheep brains by the use of a new conformation-dependent immunoassay<sup>16</sup> and supports our finding that amounts of PrP<sup>Sc</sup> will differ depending on genotype. However, the brains of 136/154/171 VVRRQQ sheep experimentally inoculated with the SSBP/1 scrapie strain had larger amounts of PrP<sup>Sc</sup> than that measured in the brains of AVRRQQ and AVRRQR sheep at the time of death. Unfortunately,

that does not substantiate our observation (using IHC) of less PrP<sup>Sc</sup> deposition in tissues of AVRRQQ inoculates than AARRQQ naturally exposed sheep with the longer incubation times. This may be a scrapie strain difference.

In the past, the observed comparison between accumulation of PrP<sup>Sc</sup>, brain lesions, and clinical signs had been taken as strong evidence for a direct neurotoxic effect of PrP<sup>Sc</sup>.<sup>17,18</sup> Certainly, when microscopic changes and neuronal loss are seen during histologic examination and large accumulations of PrP<sup>Sc</sup> are identified in neurons by IHC staining, the theory is easy to accept. However, wild-type mice can accumulate high concentrations of PrP<sup>Sc</sup> and have little if any brain changes, suggesting that the molecule may not be the neurotoxic factor.<sup>17</sup> On the other hand, results of the IHC test for detection of PrP<sup>Sc</sup> in tissue have been used to diagnose numerous cases of scrapie in sheep that had clinical signs but did not have the typical brain lesions that had formerly been required for a positive diagnosis.<sup>19</sup> Indeed, bovine spongiform encephalopathy infectivity has been transmitted in mice despite the absence of detectable abnormal prion protein.<sup>17</sup> Other cases of lightly scattered deposits of PrP<sup>Sc</sup> with few brain lesions have also been detected in sheep with severe clinical signs.<sup>20</sup> Therefore, clinical signs and death do not correspond well with brain lesions or the concentration of PrP<sup>Sc</sup>. Thus, whether PrP<sup>Sc</sup> is a cause or an effect is not known. The observation that shorter incubation time and duration of clinical signs equate to more lightly scattered PrP<sup>Sc</sup> supports the idea that PrP<sup>Sc</sup> may actually be an effect of the disease, and time is required for abnormal prion to accumulate. This is not a novel idea; several hypotheses have been put forth that bacteria, viruses, or other factors, not abnormal prions, are actually the causative agents of prion diseases.<sup>21-25</sup>

The oral route was chosen because it is the natural route of infection, although reportedly the most inefficient experimental route for PrP<sup>Sc</sup> neuroinvasion,<sup>26</sup> making the short incubation time in these sheep even more unexpected. In our study, sheep merely had the inoculum placed superficially on the back of the tongue; however, hamsters inoculated into the tongue had the shortest incubation period for transmissible mink encephalopathy ever reported for that type of hamster.<sup>27</sup> This suggests that inoculation into peripheral tissues innervated by cranial nerves originating from the brainstem will result in a more rapid direct prion neuroinvasion of the brain. In our study, older sheep may have had small abrasions around teeth, tongue, or cheeks, but it is unlikely that neonates had such lesions. On the other hand, in lambs, large protein molecules move easily through the intestinal wall after birth for approximately 24 hours, facilitating the passive uptake of maternally derived antibody. This may have facilitated absorption of PrP<sup>Sc</sup> into the intestinal lymph tissue (Peyer's patches) and mesenteric lymph nodes and may explain why the incubation time in lambs was 3 months shorter than that in the inoculated ewes. Mesenteric lymph nodes in all of the inoculated 136 AV sheep tested positive for PrP<sup>Sc</sup>, supporting results of earlier studies<sup>28,29</sup> indicating that PrP<sup>Sc</sup> enters the body via the intestines as evidenced by the fact that



accumulation of PrP<sup>Sc</sup> is first detected in lymphoreticular tissue of the intestines and associated lymphoid tissue in the naturally occurring disease.

The dose of 5 mL of SBH was purely arbitrary. When the first sheep inoculated with SBH died quickly, we believed that the sheep had been given a large overdose. However, because the inoculated AARRQQ sheep were dying of scrapie within the usual time frame with typical clinical signs, dose likely was not a factor.

The new *Uniform Methods and Rules for Scrapie Control*<sup>11</sup> recognizes that sheep that are carriers for codon 136 V are at a high risk for scrapie infection even when they also carry codon 171 R. The definition of genetically susceptible sheep now includes AVQR ewes epidemiologically associated with 136 VV or AV-positive sheep.<sup>11</sup> Veterinarians from the USDA Animal and Plant Health Inspection Service indicate that in flocks in which scrapie-positive 171 QR sheep have been identified, all or nearly all sheep testing positive for scrapie have a 136 AV or VV codon. Epidemiologists from the USDA suggest that these data strongly support the premise of a second scrapie type in the United States in which codon 136 V is the primary determinant of susceptibility,<sup>1</sup> most likely similar to the SSBP/1 strain reported in Cheviot sheep at the NPU in Edinburgh, Ireland.<sup>30</sup> Neuropathogenesis unit Cheviot sheep infected with the SSBP/1 scrapie strain reportedly have a short (2 to 3 years) incubation gene (later determined to be VV on codon 136) for scrapie manifestation and a long (4 to 6 years) incubation genotype in sheep with codon 136 AV.<sup>31</sup> The incubation period in sheep homozygous for the 136 AA genotype is believed to be longer than the sheep's lifetime or to be totally resistant.<sup>30,31</sup> However, the 136/171 AAQQ NPU Cheviots died of scrapie when infected with another strain of scrapie (CH1641), whereas the 136/171 AARR genotype conferred resistance to that strain.<sup>32</sup>

It was hypothesized that sheep used in our study may have had another nucleotide variation of the PRNP not heretofore described, which might have affected the incubation time of the traditional scrapie strain found in the United States. Thus, brain tissue of 13 of these sheep was sent to USDA for DNA sequencing of the PRNP gene. There were no notable abnormal sequences to explain the reduced incubation time. It would seem, then, that our scrapie inoculant had a V-dependent strain or a strain similar to the SSBP/1 strain, which may have originated from the two 136 V sheep brains in the inoculum.

Therefore, there are several possible explanations for the rapid incubation time seen in sheep in the study reported here: the strain may have been the traditional strain of scrapie detected in the United States, and the incubation time may have been influenced by only the 136 genotype of the sheep; this may be a V-dependent strain of scrapie similar to the SSBP/1 strain that can also affect 136 AA sheep (ie, a new, unreported strain); or the inoculum may have contained both a traditional strain detected in the United States and a V-dependent strain of scrapie.

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**Correction: Comparison of proteoglycan and collagen in articular cartilage of horses with naturally developing osteochondrosis and healing osteochondral fragments of experimentally induced fractures**

In the report, “Comparison of proteoglycan and collagen in articular cartilage of horses with naturally developing osteochondrosis and healing osteochondral fragments of experimentally induced fractures” (*AJVR*, November 2005, pp 1881–1890) The author listed as Charles L. Malemud should have been listed as Charles J. Malemud.



# Reference Point

## What veterinary practitioners should know about scrapie

Marie S. Bulgin, DVM, MBA, DACVM, and Sharon Sorensen Melson, MS

Many practitioners do not have time to keep up with new information pertaining to TSEs and, particularly with BSE, learn much of what they know from newscasts and newspapers, which can be dubious sources of information. Reliable peer-reviewed information is available in numerous scientific journals, but most are not readily available to practitioners. Scrapie is the longest known and most widely spread disease among the TSEs and remains the model for much research regarding these diseases. Scrapie affects animals in the United States and is a reportable disease and the subject of an active eradication program. However, to the authors' knowledge, the last scientific review of scrapie was published more than 10 years ago<sup>1</sup> in a journal that is not read by numerous veterinary practitioners. As a result, many sheep producers are more knowledgeable about the disease than many veterinarians. We undertook to review TSEs, update practitioners, expose certain inaccuracies regarding what is known about the disease, and answer questions that many producers expect veterinarians to know.

The TSEs are a unique group of diseases that includes scrapie and are thought by many scientists to result from accumulation of a modified cellular protein in the brain. This abnormal protein is believed to act as a template for conversion of a normal cellular protein (prion protein; PrP<sup>C</sup>) to a modified protein (PrP<sup>Sc</sup>) in susceptible animals.<sup>2</sup> Susceptibility appears to be controlled genetically in some species (eg, sheep and humans [which contract Creutzfeldt-Jakob disease] and goats<sup>3</sup> [which contract scrapie]), whereas in bovids (which contract BSE) and deer and elk (which contract CWD), a genetic connection has not been identified.

As it accumulates, this modified cellular protein is deposited as amyloid plaque in lymphoreticular and nervous tissue, where accumulation is hypothesized to cause the signs of CNS disease associated with the various TSEs. Although some researchers do not believe

### ABBREVIATIONS

TSE	Transmissible spongiform encephalopathy
BSE	Bovine spongiform encephalopathy
CWD	Chronic wasting disease
USDA APHIS VS	USDA, Animal and Plant Health Inspection Services, Veterinary Services

that the PrP<sup>Sc</sup> protein itself causes the disease,<sup>4-6</sup> results of an experimental infectivity study<sup>2</sup> have established that it is generally a reliable indicator of the presence of the infective scrapie agent.

At present, the widely accepted theory is that, in the vicinity of the abnormal prion, PrP<sup>C</sup> is induced to undergo a conformational change in which the  $\alpha$ -helical content diminishes and the  $\beta$ -sheet content increases. Although the chemical composition of the molecule does not change, the structural difference changes its chemical properties.<sup>2</sup> For example, PrP<sup>C</sup> is soluble in denaturing detergents and is digested by cellular proteases such as proteinase K.<sup>7</sup> The PrP<sup>Sc</sup> (and its infectivity) is not destroyed by detergents, but is resistant to breakdown by rendering processes used at present, heat sterilization temperatures, UV light, ionizing radiation, and many disinfecting agents and is only partially inactivated by proteinase K.

The genetics of scrapie in sheep have been described.<sup>8</sup> Located on chromosome 13, the PrP gene (Prnp) is composed of 3 sections called exons and another section called an open reading frame. The open reading frame is 256 amino acid codons in length. A codon is a 3-nucleotide segment of DNA and is comparable to a street address. For example, codon 136 is the 136th sequence of 3 DNA molecules that code for a given amino acid. In sheep, scrapie is linked to at least 3 polymorphisms in the PrP gene that are responsible for amino acid changes in PrP<sup>C</sup> encoded by codons 136, 154, and 171.

Codon 136 codes for valine (V), alanine (A), or threonine (T); codon 154 codes for arginine (R) or histidine (H); and codon 171 codes for glutamine (Q), lysine (K), H, or R. Resistance to scrapie appears to be influenced by 136A,<sup>9,10</sup> 154H,<sup>11</sup> and 171R.<sup>12</sup> Genotypes 136T, 171K, and H are apparently rare worldwide, and 136V and 154H are also rare in the United States.<sup>12</sup> Genotypes 136V and 171Q appear to be linked because 136V is never found concurrently with 171R. These small changes in the amino acid components of this

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glyco-plasma membrane protein enable it to resist re-configuration.

The purpose of the prion protein has only recently begun to be determined. Laboratory rodents that are devoid of prion protein have been developed, and no deleterious effects of that mutation have been detected.<sup>a</sup> The fact that the prion protein is found in most animal species and is highly conserved suggests that it has an important function. Nevertheless, prion-free knockout rodents appear to be clinically normal and have complete resistance to TSEs. A recent study,<sup>b</sup> however, revealed that irradiated mice that received repeated transfers of prion-free bone marrow stem cells became less hardy and weaker with each subsequent transplant. Stem cells from clinically normal mice containing prion protein established themselves in irradiated mice, which survived subsequent sequential transplantations and remained healthy. Prion protein appears to protect cells from stress resulting from multiple replications. Furthermore, in cell culture, it appears to protect a breast cancer cell line against tumor necrosis factor- $\alpha$ -mediated cell death.<sup>13</sup> It also appears to promote cellular survival *in vivo* under certain conditions and to have superoxide dismutase (antioxidant) and neuroprotective properties related to uptake of divalent copper.<sup>13</sup>

The prion protein is not identical among the various animal species affected by TSEs. However, there is sufficient similarity that multiple antibodies bind to prion proteins from different species. These multispecies antibodies are useful for screening tests.<sup>14</sup> Hence, the same tests are used for detection of BSE, scrapie, and CWD in deer and elk. So far, all diagnostic tests for TSEs function on the basis of serum antibody reactions. Because animals generally do not make antibodies against self, antibodies are mostly monoclonal in origin or are made in knockout rodents that lack prion protein. Most of these antibodies react against both normal and abnormal prions, and positive test results generally rely on the ability of the abnormal prion to withstand denaturing detergents, heat, and proteinase K digestion.<sup>7</sup>

Diagnostic tests commonly used for identification of scrapie are immunohistochemical testing, Western blot analysis, and ELISAs. At least 4 ELISAs are used at present in the European Union for scrapie surveillance at slaughter.<sup>15,c</sup> None of those assays are validated for use in the United States for diagnosis of scrapie, although several ELISA test kits have been licensed by the USDA Center for Veterinary Biologics for diagnosis of CWD in wild elk and deer.<sup>16</sup> The immunohistochemical test is considered to be the gold standard for testing in the United States, and it is also the confirmatory test at some labs of the World Organization for Animal Health.<sup>17</sup> The Western blot test is used primarily as a research tool and confirmatory test for BSE. An early report<sup>18</sup> on an immunocapillary electrophoresis assay indicated that it might be a valuable tool for detection of human BSE (ie, new-variant Creutzfeldt-Jakob disease) and scrapie in blood, but it is still undergoing evaluation as a diagnostic tool. Recently, other blood tests have been described that involve more sophisticated techniques such as protein misfolding cyclic amplification, aggregation-specific ELISA, and fluorescent

amplification catalyzed by 17-RNA polymerase. All of these tests depend on amplification or replication of the prion many thousands of times.<sup>19</sup> The latter test can detect target protein at femtomolar concentrations and can also detect naturally infected mule deer with CWD and scrapie-inoculated mice with no clinical signs with 100% sensitivity and specificity.

**The disease**—At the time of manuscript preparation, the prevalence of scrapie in the United States was 0.1% to 0.3% (percentages that decreased from approx 0.5% before the eradication program began), as determined by slaughter surveillance.<sup>20</sup> Scrapie primarily affects blackface sheep breeds (eg, Suffolk, Hampshire, and crosses of those breeds), which account for approximately 96% of the overall prevalence of the disease.<sup>20</sup> In the United States, the disease is chiefly related to polymorphisms at codon 171 because the 136AA and 154RR genotypes are reportedly the norm among US sheep.<sup>20,21</sup> Ninety-one percent of brain samples with positive test results for scrapie originated from sheep with the AARRQQ genotype.<sup>20</sup> However, individual flocks may have high prevalences of sheep with the 136V genotype,<sup>22,23</sup> and genotype 154H is also occasionally detected. Given the predominance of scrapie in the Suffolk breed, many producers believe that Suffolk sheep are more susceptible to developing scrapie than other breeds, but this has not been confirmed. Although genotyping of 2,300 blackface sheep (primarily Suffolks) in a 2003 report<sup>12</sup> revealed that 43% of those sheep were of the 171QQ genotype and only 13% were of the 171RR genotype, other domestic sheep breeds have a higher frequency of susceptible genetics. Columbia sheep, for example, are predominantly of the 171QQ genotype.<sup>21</sup>

The high prevalence of scrapie in Suffolks in the United States is likely to be related to other factors, including that the disease was imported in Suffolk sheep; imported animals are expensive, and expensive sheep genetics are typically not wasted on crossbred or inferior flocks but rather remain congregated in purebred flocks; if crossbreeding is done, males are usually bred to females of different breeds and the disease is not transmitted by males; and Suffolk sheep may be used surreptitiously to confer desirable traits to sheep of Hampshire breeding, which has the next highest prevalence (5%) of the disease. Sheep of whiteface breeds (eg, Rambouillet, Southdown, and Cheviot) have also developed scrapie,<sup>20</sup> but most had a history of contact or likely contact with Suffolks. So far, scrapie has not been diagnosed in whiteface sheep grazed on public lands in the western United States.

The 136/171 AAQQ genotype has been associated with all instances of scrapie reported in the United States except for a few sheep with the 171QR genotype, which, to date, has been associated with the 136AV genotype.<sup>20</sup> The 171QR genotype is considered to confer resistance to scrapie, particularly when coupled with 136AA. In the United Kingdom, the 136/154/171 AARRQR genotype is one of the most resistant genotypes.<sup>24</sup> That genotype has been detected in 28% of sheep in the United Kingdom, but the prevalence of the disease there is only 0.4 cases/million head of sheep.



In the new *Uniform Methods and Rules for Scrapie Control*<sup>25</sup> published by the USDA, it is recognized that sheep with codon 136V are at high risk for developing scrapie even when they also carry codon 171R. The definition of genetically susceptible sheep now includes ewes with the 136/171 AVQR genotype that are epidemiologically associated with flocks in which scrapie has been diagnosed in sheep with the 136VV or 136AV genotypes.

In the United States and European Union, efforts to control and eliminate the disease by selecting sheep with at least one 171R codon and removing sheep with genotype 171QQ are in progress,<sup>25,26</sup> with the expectation that the remaining sheep will be resistant to scrapie infection. In the states of Texas<sup>27</sup> and Washington,<sup>28</sup> breeding animals entering the state must be of the 171RR or 171QR genotypes. Veterinarians from the USDA APHIS VS require elimination of all sheep with the 171QQ genotype from flocks affected with scrapie before lifting a flock quarantine, but allow sheep with the QR genotype to remain unless associated with codon 136AV.<sup>25</sup> Skeptics of this scheme worry that reliance on genetics for elimination of the disease may induce a false sense of security given that there have been several reports<sup>29-31</sup> of scrapie in Suffolk sheep with the 171RR genotype. However, the genotype susceptibility differences seem to be related to scrapie strain differences.

In 1988, Irish researchers using scrapie strain SSBP/1 reported<sup>32,33</sup> that Cheviot sheep had a short-incubation (2 to 3 years) genotype for scrapie manifestation, which was later found to be codon 136VV. Those investigators also found that sheep with the 136AV genotype had a long-incubation genotype (4 to 6 years). Sheep homozygous for the 136AA genotype were resistant or had an incubation period that was longer than the sheep's lifetime. When another scrapie strain (CH1641) was inoculated into Cheviot sheep with the 136/171 AAQQ genotype, however, those sheep were susceptible to scrapie, whereas sheep with the 136/171 AARR genotype were resistant to the CH1641 strain. Furthermore, the 171RR codon also seemed to convey partial resistance to experimentally induced BSE in sheep that underwent intracerebral inoculation.<sup>33,d</sup>

In flocks containing scrapie-infected sheep that carry the 171QR genotype, all or nearly all sheep with positive results of tests for scrapie also have the 136AV or 136VV genotype. Epidemiologists from the USDA APHIS VS suggest that these data strongly support the possibility that there is a second scrapie type in the United States in which codon 136V is the primary determinant of susceptibility; that strain has been referred to as a V-dependent strain.<sup>e</sup>

Strains of scrapie specific to the United States are unknown. However, because CH1641, the prototype for strain C, causes disease in sheep of the 171QQ genotype regardless of the 136 genotype, some researchers hypothesize that the C strain is the most prevalent US strain; it may be reasonable to suppose that SSBP/1, the prototype for scrapie strain A, would be the V-dependent strain in this country. Strain differentiation, however, requires comparison of the biochemical properties of the PrP<sup>Sc</sup> or strain typing by mouse bioassay and lesion-profile scoring performed by inoculating 3 con-

ventional mouse lines.<sup>34</sup> The mouse bioassay is arduous and time-consuming, and its use has not been reported to the author's knowledge for US scrapie strains.

Nor98, a more recently discovered strain, was detected during surveillance testing by 1 of the 4 ELISAs<sup>f</sup> that are presently used in Europe as rapid screening assays.<sup>35-37,g</sup> The strain was originally reported in 5 unrelated Norwegian sheep with clinical signs that had the unusual genotype of 136/154/171 AAHHQQ or AAHRQQ, genotypes that are typically associated with resistance conferred by the Hat codon 154. In those sheep, no PrP<sup>Sc</sup> was found in either lymphoid tissues or at the level of the obex, but was detected primarily in the cerebellum. Results of Western blot analysis indicated that the glycoform was different from other known scrapie strains or BSE and had a distinctive PrP<sup>Sc</sup> electrophoresis profile that included a low-molecular-weight protein band of 12 kDa.<sup>35</sup> More recently, a double leucine or leucine-phenylalanine polymorphism at a newly reported site, codon 141, has also been associated with some of these cases.<sup>37</sup> As of June 2004, 38 sheep with scrapie involving this strain had been identified.

More worrisome are the findings of other atypical cases of scrapie in the European Union. An obligatory active surveillance program was implemented there in 2002 that was based on large-scale testing of both slaughtered and recumbent small ruminants and that led to a considerable increase in the number of animals with a diagnosis of scrapie. The program has also revealed a number of cases of so-called discordant disease. Interestingly, these cases have also been detected with only 1<sup>l</sup> of the 4 screening ELISAs. In some of these discordant cases, the low-molecular-weight band associated with Nor98 was detected and some of the involved sheep were homozygous for 171R.<sup>30,31</sup> Moreover, by the beginning of 2005, the Veterinary Laboratories Agency of Great Britain had detected 83 atypical cases of scrapie from 110,000 tested samples. Of those atypical cases, 12 sheep had the RR genotype, which is associated with the greatest resistance to scrapie.<sup>31</sup> No explanation has been given for the increased sensitivity<sup>b</sup> of that ELISA for detection of atypical cases of scrapie, but the anti-prion antibody used in the test may play a role.

The Nor98 strain and other cases of discordant scrapie involved single sheep in flocks with no obvious contact with affected flocks, suggesting that the infectious agent may not have been transmitted by direct contact.<sup>38</sup> It has been hypothesized that such cases represent spontaneous prion disease, analogous to sporadic Creutzfeldt-Jakob disease in humans, and that the affected sheep were not infectious. However, the disease has been transmitted from samples of Nor98 infected brain and the brains of 3 other sheep with discordant scrapie (genotype 171RR) to transgenic mice expressing sheep prion protein.<sup>37</sup> It is also possible that these scrapie strains are similar to BSE in that they do not appear to be transmitted by natural means. Nevertheless, it is of concern that sheep with the 136/154/171 AARRRR genotype can no longer be assumed to be free of naturally acquired TSE. This finding challenges the foundation of selective breeding programs sponsored by the USDA APHIS VS and certain members of the European Union. In March 2007, the USDA announced that it had identified a single Wyoming ewe with Nor98 strain of scrapie; however, other discordant strains have not yet been found in the United



States. Unless the USDA APHIS VS endorses use of the ELISA test that has successfully detected the atypical scrapie strains, it is not likely that we will know what the prevalence of these odd strains in the United States really is.

**Disease transmission**—Interestingly, scrapie does not appear to be transmitted in utero despite the fact that the placenta and placental fluids contain PrP<sup>Sc</sup>. Lambs delivered via Caesarian section from infected dams and isolated from infected sheep remain disease free.<sup>39</sup>

Despite the wide distribution of PrP<sup>C</sup> in reproductive, placental, and certain fetal tissues and fluids, PrP<sup>Sc</sup> has been detected only in the caruncular portion of the endometrium and cotyledonary chorioallantois (the fetal-maternal interface) of pregnant scrapie-infected ewes<sup>40</sup> and only if both dam and fetus are of a susceptible genotype.<sup>41</sup> The embryo or fetus is not exposed to scrapie while in utero in a scrapie-infected dam because there is physical separation from PrP<sup>Sc</sup>-containing allantoic fluid and chorioallantois by the amnion, which remains free of PrP<sup>Sc</sup> even when the other placental tissues are infected.<sup>41,42</sup> In other words, even if tissues of the maternal side of the placenta carry a susceptible prion protein, susceptible prion-containing cells from the fetal side of the placenta appear to be necessary for conversion to PrP<sup>Sc</sup>. Thus, an infected ewe introduced into a clean flock and bred to a ram with the 171RR genotype is unlikely to transmit scrapie. That does not necessarily hold true, however, if the ewe is bred to a ram with the 171QR genotype. In that instance, the spatial relationship between fetuses in utero can influence PrP<sup>Sc</sup> accumulation in ewes carrying fetuses with different genotypes. It appears that partial or incomplete anastomosis can exist in the blood supply to cotyledons of fetuses of different genotypes on the same side of the uterine horn,<sup>42</sup> and this can result in accumulation of PrP<sup>Sc</sup> in cotyledons with resistant genotypes.

The disease is naturally transmitted from infected dams during lambing via ingestion of infected placenta or allantoic fluids by flockmates and newborn lambs. Infected males are not believed to transmit the disease.

Findings from earlier research suggested that other body excretions remain free of scrapie infectivity. However, it is now known that scrapie can be transmitted to other sheep via blood transfusion,<sup>43</sup> and antemortem detection of scrapie prions in the blood has recently been reported.<sup>19</sup> Infectivity via that route is low, however, with a large volume of blood (400 to 500 mL) required to transmit disease.

Replication of scrapie prion requires the participation of cells related to the immune system (such as follicular dendritic cells and B-lymphocytes expressing tumor necrosis factor) and lymphotoxin  $\alpha$  and  $\beta$  (factors present in the spleen and lymph nodes).<sup>44</sup> Chronic inflammatory conditions are usually accompanied by accumulation of B and T lymphocytes, follicular dendritic cells, and lymphotoxins at the site of inflammation. In 1 study<sup>45</sup> involving mice with chronic hepatitis, nephritis, and pancreatitis, mice infected with scrapie were found to have PrP<sup>Sc</sup> in the liver, kidneys, and pancreas, organs that do not contain scrapie prion in typical scrapie-infected mice. In another study,<sup>46</sup> naturally infected sheep with scrapie were also infected with mae-

di-visna virus, the European strain of ovine progressive pneumonia virus. In that study, PrP<sup>Sc</sup> was found in the mammary gland of sheep with lymphoid follicular mastitis (ie, hard bag), a common clinical sign of infection with the ovine progressive pneumonia virus. A common disease of sheep in the United States, ovine progressive pneumonia is characterized by lymphocytic infiltration of the lungs and mammary gland; joints and brain tissue may also be affected. Weight loss is a common sign of this disease, and when the brain is affected (the condition called visna), signs can mimic those of scrapie.

It is widely accepted that previous premises contamination can be a source of scrapie infection. Anecdotal accounts abound of flock depopulation and premises decontamination, followed by recurrence of disease in repopulated infection-free sheep. An early experiment<sup>46</sup> in which scrapie-contaminated material was buried for 3 years indicated that it remained infectious when unearthed.<sup>47</sup> In another study,<sup>48</sup> it was found that prions released in soil rich in phyllosilicates (such as clay) would be strongly adsorbed but could remain active. This may increase the risk of infection for ruminants grazing contaminated pastures or exposed to contaminated groundwater. Researchers believe that the infectious agent of scrapie has persisted in regions of Iceland for at least 16 years,<sup>49</sup> and its persistence has been proposed as being related to high concentrations of iron and a high iron-to-manganese ratio in the forage of those areas.<sup>50</sup> Carriage of prions by grass mites has also been reported,<sup>51</sup> but the importance of this finding is unknown.

**Disease signs**—Classic scrapie, which results from ingestion of PrP<sup>Sc</sup> by a susceptible sheep carrying the 136/171AAQQ genotype (the most common genotype in the United States), is a long-term, progressive, and debilitating neurologic illness that is believed to be uniformly fatal. Clinical signs may be noticed from 18 months to 5 years after exposure and include progressive weight loss with no concurrent loss in appetite, progressive ataxia, fine head tremors (most apparent in the ears), and cutaneous hypersensitivity. In an earlier study,<sup>23</sup> only about 70% of naturally exposed sheep with clinical signs of scrapie had pruritis (the clinical signs of which give rise to the disease's name); pruritis was not observed in any of the experimentally inoculated sheep with the AVQQ genotype.<sup>23</sup> Behavioral changes are often detected, with sheep assuming a vacant, fixed stare or suddenly becoming aggressive. Signs of hypersensitivity are often elicited by rubbing or scratching the sheep's back, which induces the sheep to throw its head back, make chewing motions and lick at the air, or compulsively nibble at the limbs below the carpus.

Ataxia is first detected when sheep are running. The hind limbs of sheep appear to be uncoordinated with the forelimbs, and affected animals adopt a bunny-hopping gait. Sheep often have a high-stepping gait in the forelimbs, resembling a prancing horse. As signs worsen, the hindquarters may sway when the sheep is standing.

Clinical signs last from 1 to > 3 months; sheep generally become recumbent because of weakness and incoordination. If helped up, an affected sheep may be able to remain standing for hours, but may not be able



to rise unassisted if it falls or lies down. Death follows within 1 to 2 weeks of a sheep becoming unable to right itself. Blindness, resembling that seen with polioencephalomalacia, occasionally develops.

Differential diagnoses include other diseases characterized by chronic weight loss: caseous lymphadenitis, abomasal emptying disease, Johne's disease, ovine progressive pneumonia (visna), dentition problems, and meningitis. The clinical signs of scrapie can vary, depending on the sheep's genotype and the strain of scrapie involved. Sheep with genotype 136/154/171 AVRQQ that were orally inoculated with a brain homogenate from 7 domestic scrapie-infected sheep died after an abnormally short incubation time of approximately 1 year.<sup>23</sup> In those sheep, clinical signs varied from none (ie, sudden death) to being found unable to rise in the absence of previous ataxia and dying in 2 to 3 days to a substantially more rapid clinical course of the classic manifestation, lasting no longer than 3 weeks. Weight loss and signs of pruritis were not observed. Most veterinarians or producers would not recognize sheep with these signs as having scrapie. A complete necropsy should be performed on any sheep that dies unexpectedly, including evaluation and submission of the brain for immunohistochemical testing for scrapie.

**Diagnosis**—The pathologic changes associated with scrapie are confined to the CNS and include vacuolation, neuronal loss, astrocytosis, and accumulation of amyloid plaques. However, because histologic change can be inapparent, diagnosis of the disease in the United States relies on immunohistochemical testing to reveal the presence of PrP<sup>Sc</sup> in brain or lymphoid tissue.<sup>52</sup> The necessity for this type of testing was particularly evident in the study<sup>23</sup> involving inoculated 136/171 AVQQ-genotype sheep. Sheep do not mount an immune response against the abnormal prion, and at present, no sensitive or completely reliable test for diagnosis during the preclinical stages of the disease is available.

Detection of PrP<sup>Sc</sup> in spleen; retropharyngeal, mesenteric, and prescapular lymph nodes<sup>53</sup>; third-eyelid lymphoid tissue<sup>54</sup>; tonsil<sup>55</sup>; tongue<sup>56</sup>; retina<sup>57</sup>; rectal ring tissue<sup>58</sup>; spinal fluid<sup>59</sup>; and blood<sup>19,60</sup> prior to appearance of PrP<sup>Sc</sup> in the brain or of clinical signs has been reported. Deposition of PrP<sup>Sc</sup> in certain tissues enables detection of subclinical disease by biopsy in some instances. Detection of PrP<sup>Sc</sup> has been reported<sup>1b</sup> in 76% of tonsils examined and 57% of lymphoid tissue specimens collected from the third eyelid of infected sheep. A small number of sheep in which the brain contains PrP<sup>Sc</sup> do not have detectable PrP<sup>Sc</sup> in the lymph nodes, and that number may be influenced by sheep genotype or scrapie strain.<sup>35,61</sup> The Nor98 strain, for example, has not been found in lymphoid tissues.<sup>35</sup>

The palatine tonsil has been used for biopsy and diagnosis,<sup>55,62</sup> but obtaining samples is not practical for use in live animals. Biopsy of lymph follicles of the third eyelid, although simpler and validated as a diagnostic test, yields a high percentage of unreadable samples because follicles may not be present in adequate numbers in as many as 40% to 60% of adult sheep.<sup>h</sup> Biopsy of other lymphoid tissues, such as mandibular lymph nodes and rectal mucosa, has not been validated as a

diagnostic technique. However, biopsy of those sites may be useful diagnostically because examination of several tissues is likely to improve the chances of diagnosis. It has not been established at what time in the course of the disease the agent will consistently appear in these tissues, but it may be as early as 14 months after exposure. This interval likely depends on the age of the sheep at exposure and on the genotype or strain of scrapie because the incubation time in sheep with the 136V genotype appears to be shorter.<sup>23</sup>

Thorough histologic examination of the third eyelid has revealed that most lymphoid follicles are in the lamina propria of protuberances on the palpebral surface, rather than on the bulbar surface. Instillation of histamine into the conjunctival sac temporarily inflames the lymphoid follicles and may make them easier to sample. Administration of xylazine (0.1 mg/lb, IV) for restraint and topical administration of an anesthetic facilitates biopsy of third-eyelid lymphoid tissue and enables easy sampling of the mandibular lymph node and rectal-anal mucosa.

Regarding rectal biopsy, lymphoid tissue is located between the folds of the entire circumference of the rectal mucosa, extending to a point approximately 1 cm cranial to the mucocutaneous junction at the entry to the rectum. A small specimen of this tissue can easily be obtained from even an unsedated sheep.<sup>58,62</sup> Lymphoid tissue collected from either the eyelid or rectum should be laid on a small piece of sponge to keep it flat; sponge and adherent tissue can be placed in formalin solution without the tissue curling. For disinfection, instruments should be soaked in 2.5N NaOH or another disinfectant with activity against abnormal prions<sup>63,i</sup> for at least 24 hours before rinsing and sterilization in an autoclave.

In summary, scrapie is a reportable disease with an active and ongoing eradication program. The disease may cause clinical signs that are subtle and nonspecific, but most sheep with scrapie have one or more of the following signs: pruritis, weight loss without appetite loss, ataxia, weakness, head tremors, and cutaneous hypersensitivity. Early clinical signs may be restricted to fine tremors of the head that progress during a 1- to 3-month interval to noticeable head tremors, incoordination, and recumbency. Diagnosis is made on the basis of results of immunohistochemical staining of the obex and other parts of the brain or lymphoid tissue for PrP<sup>Sc</sup>. At present, no highly sensitive test is available for use in live animals; however, biopsy of lymphoid tissue from the third eyelid, rectum, or mandibular lymph node may reveal sheep with scrapie before clinical signs develop. Recent development of a test capable of detecting PrP<sup>Sc</sup> in blood<sup>60</sup> yields hope that a simple, cost-effective, and sensitive blood test for preclinical diagnosis of scrapie will be available in the future.

In the United States, sheep of blackface breeds (chiefly Suffolks) are responsible for 96% of scrapie diagnoses. Whiteface sheep comprise only a small percentage (4%) of cases, and the disease has yet to be reported in whiteface range sheep in the western United States. Genetics play a role in the susceptibility of sheep to scrapie, with the codons 136, 154, and 171 being chiefly involved. Sheep with codon 136VV genotypes are more susceptible to scrapie than those with



the 136AV genotype; sheep with the 136AA genotype are less susceptible. Sheep with codon 171RR are the most resistant to scrapie, followed by sheep with the QR genotype. Sheep with the QQ genotype are the least resistant. Codon 154H confers resistance, but is rare in US sheep. At least 12 sheep with the 171RR genotype have been diagnosed with scrapie in the European Union on the basis of an ELISA test that is not presently used in the United States.

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## Short Communication

## Communication brève

## Experimental transmission of scrapie agent to susceptible sheep by intralingual or intracerebral inoculation

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### Abstract

Scrapie, a transmissible spongiform encephalopathy (TSE), is a naturally occurring fatal neurodegenerative disease of sheep and goats. This study documents survival periods, pathological findings, and the presence of abnormal prion protein (PrP<sup>Sc</sup>) in genetically susceptible sheep inoculated with scrapie agent. Suffolk lambs (AA/RR/QR at codons 136, 154, and 171, respectively) aged 4 mo were injected by the intralingual (IL) or intracerebral (IC) route with an inoculum prepared from a pool of scrapie-affected US sheep brains. The animals were euthanized when advanced clinical signs of scrapie were observed. Spongiform lesions in the brain and PrP<sup>Sc</sup> deposits in the central nervous system (CNS) and lymphoid tissues were detected by immunohistochemical and Western blot (WB) testing in all the sheep with clinical prion disease. The mean survival period was 18.3 mo for the sheep inoculated by the IL route and 17.6 mo for those inoculated by the IC route. Since the IC method is occasionally associated with anesthesia-induced complications, intracranial hematoma, and CNS infections, and the IL method is very efficient, it may be more humane to use the latter. However, before this method can be recommended for inoculation of TSE agents, research needs to show that other TSE agents can also transmit disease via the tongue.

### Résumé

La tremblante fait partie des encéphalopathies spongiformes transmissibles (TSE) et est une maladie neuro-dégénérative fatale naturelle rencontrée chez les moutons et chèvres. La présente étude fait état des résultats obtenus quant aux périodes de survie, aux trouvailles pathologiques et à la présence de protéines prions anormales (PrP<sup>Sc</sup>) chez des moutons génétiquement susceptibles inoculés avec l'agent de la tremblante. Des agneaux Suffolk (AA/RR/QR aux codons 136, 154 et 171, respectivement) âgés de 4 mo ont été injectés par voie intra-linguale (IL) ou intracérébrale (IC) avec un inoculum préparé à partir d'un pool de cerveaux de moutons souffrant de tremblante provenant des États-Unis. Les animaux ont été euthanasiés lorsque des signes cliniques avancés de tremblante étaient observés. Les lésions spongiformes dans le cerveau et des dépôts de PrP<sup>Sc</sup> dans le système nerveux central (CNS) et les tissus lymphoïdes ont été détectés par immunohistochimie et immuno-buvardage (WB) chez tous les moutons présentant une maladie à prion clinique. La période moyenne de survie était de 18,3 mois pour les moutons inoculés par la voie IL et de 17,6 mois pour ceux inoculés par la voie IC. Comme la méthode d'inoculation IC est parfois associée avec des complications dues à l'anesthésie, des hématomes intracrâniens et des infections du CNS, et que la méthode IL est très efficace, il serait plus éthique d'utiliser cette dernière. Toutefois, avant que cette méthode ne soit recommandée pour l'inoculation d'agents de TSE, les recherches doivent démontrer que d'autres agents de TSE peuvent également être transmis via la langue.

(Traduit par Docteur Serge Messier)

Scrapie belongs to a group of diseases known as transmissible spongiform encephalopathies (TSEs). It is a naturally occurring, genetically influenced, fatal neurodegenerative disease of sheep and goats. Infection by the causative agent, considered to be the post-translationally modified form of the host-encoded membrane-bound prion protein (PrP<sup>C</sup>), leads to spongiform encephalopathy associated with accumulation of the abnormal form of prion protein (PrP<sup>Sc</sup>) in tissues of the nervous and lymphoid systems, as well as in the placenta (1).

The most likely portal of entry in natural scrapie has been suggested to be the alimentary tract; other potential portals, such as scarified skin or the conjunctiva, have been effective experimentally (1). There is a paucity of information on experimental studies with scrapie in Suffolk sheep, the dominant sheep breed in the

United States. In particular, the various routes of infection, other than oral and intracerebral (IC) with the US scrapie agent (2), have not been documented previously. This study attempted to partially fill this void by comparing intralingual (IL) and IC administration of the US scrapie agent to genetically susceptible Suffolk sheep.

Nine 4-mo-old Suffolk lambs (4 females and 5 castrated males) were obtained from a scrapie-free sheep flock at the National Animal Disease Center (NADC), Ames, Iowa. All were AA/RR/QR at codons 136, 154, and 171, respectively, of the PRNP gene. The animals were divided into 2 groups: 4 lambs received the scrapie inoculum by the IL route, and 5 lambs received it by the IC route.

The inoculum (X124) was prepared from a pool of 7 scrapie-affected sheep brains from a single flock (3). All 7 sheep were QR at codon 171 of the PRNP gene, and their brains were positive by

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**Table 1. Findings consistent with scrapie in tissues of susceptible Suffolk sheep injected with US scrapie agent X124 by the intralingual (IL) or intracerebral (IC) route**

Sheep ear-tag no., sex	Survival period <sup>a</sup>	Histologic lesions of SE/PrP <sup>Sc</sup> by IHC					PrP <sup>Sc</sup> by IHC			PrP <sup>Sc</sup> by WB; BS
		O	C	SC	H	RC	R	LN	T	
<b>IL route</b>										
3511, F <sup>b</sup>	9 d	-/-	-/NE	-/NE	-/NE	-/NE	NE	NE	NE	-
3513, M	18 mo	+/+	+/+	+/+	-/+	+/+	+	+	+	+
3514, F	19 mo	+/+	+/+	+/+	-/+	+/+	+	+	+	+
3502, M	18 mo	+/+	+/+	+/+	-/+	-/+	+	+	+	+
<b>IC route</b>										
3501, F	15 mo	+/+	+/+	+/+	+/+	+/+	+	+	+	+
3510, M	16 mo	+/+	+/+	+/+	+/+	+/+	+	+	+	+
3512, M	15 mo	+/+	+/+	+/+	+/+	+/+	+	+	+	+
3509, F	15 mo	+/+	+/+	+/+	-/+	+/+	+	+	+	+
3504, M	26 mo	+/+	+/+	+/+	+/+	+/+	NE	-	+	+

SE — spongiform encephalopathy; PrP<sup>Sc</sup> — abnormal prion protein; IHC — immunohistochemical testing; O — obex; C — cerebellum; SC — superior colliculi; H — hippocampus; RC — rostral cerebrum; R — retina; LN — retropharyngeal lymph node; T — tonsil (palatine and pharyngeal); WB — Western blot testing; BS — brainstem; NE — not examined.

<sup>a</sup> Time between inoculation and euthanasia.

<sup>b</sup> This lamb did not have signs of scrapie; it was euthanized at 9 d for detection of inoculated material in the tongue.

<sup>c</sup> Only the palatine tissue was positive.

Western blot (WB) analysis. The brains were sonicated, and a final concentration of 10% (w/v) was prepared with phosphate-buffered saline. The animals were each injected with a standard 1 mL of the inoculum.

For the IL route, the lambs were not given any sedation. The inoculum was injected with a 20-gauge needle into the ventral aspect of the tongue, approximately 2.5 cm from the tip. The IC method has been described previously (4). Briefly, the lambs were sedated with xylazine, a midline incision was made in the skin at the junction of the parietal and frontal bones, and a 1-mm hole was trephined through the calvarium. The inoculum was injected into the midbrain with a 22-gauge, 9-cm-long needle as the needle was withdrawn from the brain. The skin incision was closed with a single suture.

The inoculated animals were housed separately in a biosafety level 2 containment facility for 2 wk and then moved to 2 outside pens at NADC. They were fed pelleted growth and maintenance rations that contained no ruminant protein, and clean water was freely available.

One lamb, injected by the IL route, was euthanized 9 d after inoculation for immunohistochemical (IHC) detection of inoculated material in the tongue tissue; at necropsy, only the brain (for negative-control samples) and the tongue were obtained. The other 8 animals were euthanized when advanced clinical signs of scrapie developed. In these 8, a detailed gross examination did not show any lesions. Two sets of representative tissue samples were collected. One set of tissues included representative sections of liver, kidney, spleen, skin, striated muscles (heart, tongue, diaphragm, masseter), tonsils (pharyngeal, palatine), thyroid gland, turbinates, lung, intestines (ileum), adrenal gland, lymph nodes (retropharyngeal, mesenteric), pituitary gland, Gasserian ganglion, brain (hemisections of cerebral cortex, cerebellum, superior colliculi, and brainstem, including obex), and eye (retina). For the tongue, a minimum of 8 cross-sections from

the site of inoculation of each animal that received an IL injection were obtained. These tissues were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin (H-E) for light microscopy. The 2nd set of tissues was frozen.

All paraffin-embedded tissues and negative-control sections were labeled by an automated IHC method for detection of PrP<sup>Sc</sup> as described previously (2). For WB detection of PrP<sup>Sc</sup> in the brainstem, a commercial kit (Bio-Rad, Marnes-La-Coquette, France) with monoclonal antibody P4 was used as described previously (5).

Except for the lamb euthanized 9 d after inoculation, the animals showed clinical signs of scrapie before euthanasia. Initial signs were a progressive decrease in appetite and associated weight loss. Later signs were fine head tremors, listlessness, progressive problems with locomotion, and terminal sternal recumbency. None of the sheep exhibited obvious pruritus or loss of wool.

Table 1 shows the survival period after inoculation for the individual animals. The mean was 18.3 mo for the group inoculated by the IL route and 17.6 mo for the group inoculated by the IC route; analysis of variance did not show a significant difference ( $P = 0.76$ ). Table 1 also shows the distribution of histologic, IHC, and WB findings. In all the sheep except the lamb euthanized 9 d after inoculation (sheep 3511), microscopic spongiform lesions characteristic of prion disease (Figure 1) were observed, and characteristic PrP<sup>Sc</sup> deposition was present in the CNS samples. In addition, PrP<sup>Sc</sup> deposition was present in the retropharyngeal lymph node and the tonsils (Figure 2), as well as all the tested retinas (Figure 3), of the sheep with clinical scrapie. A single submucosal lymphoid aggregate was detectable in the 3rd-eyelid samples from 1 of the sheep with an IHC-positive brain; however, this follicle contained no detectable PrP<sup>Sc</sup>. Submucosal lymphoid follicles were not present in the other examined 3rd eyelids.



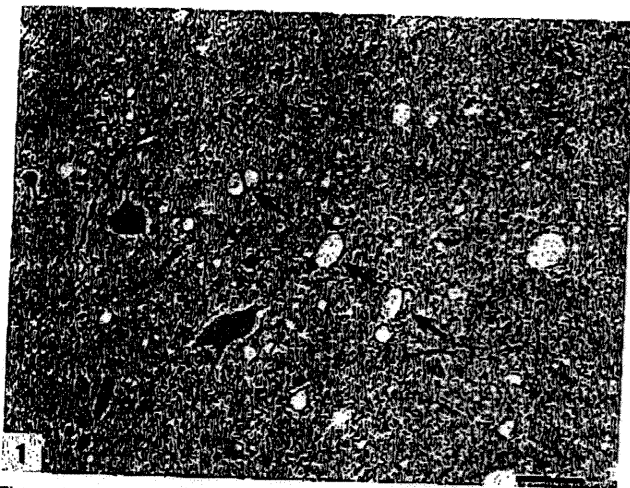


Figure 1. Medulla oblongata at the obex level of sheep 3502, with clinical scrapie, showing extensive vacuolation of the neuropil and multiple vacuoles in the neuronal perikaryon (arrows). Note the paucity of normal-appearing neurons. Hematoxylin and eosin (H-E). Bar — 30  $\mu$ m.



Figure 3. Retina of sheep 3502, showing extensive diffuse PrP<sup>Sc</sup> labeling in both inner and outer plexiform layers. Bar — 20  $\mu$ m.



Figure 2. Pharyngeal tonsil of sheep 3502, showing immunohistochemical (IHC) staining (red) of the abnormal form of prion protein (PrP<sup>Sc</sup>), which is mainly in the germinal centers of follicles. Immunohistochemical phosphatase and (counterstain) hematoxylin. Bar — 250  $\mu$ m.

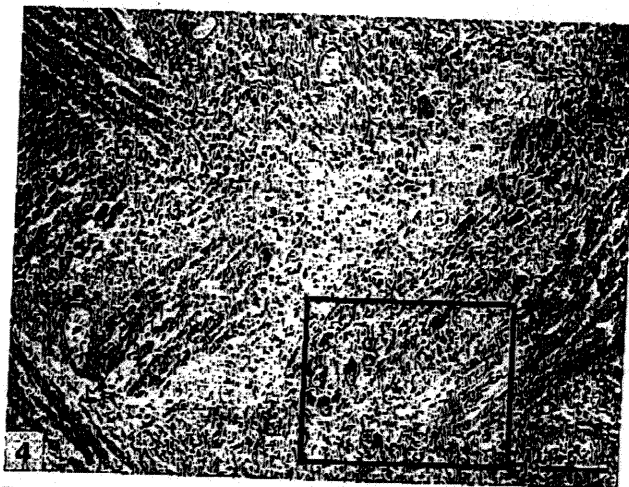


Figure 4. Tongue of sheep 3511, euthanized 9 d after inoculation with scrapie agent X124, showing extensive infiltration of epithelioid macrophages and lesser numbers of multinucleated giant cells between muscle fibers. H-E. Bar — 100  $\mu$ m.

In sheep 3511, a locally extensive cellular infiltrate was noted at the site of inoculation in the tongue. The inflammatory cells were predominantly epithelioid macrophages arranged in broad sheets (Figure 4). At scattered sites within the infiltrate there were small numbers of multinucleated giant cells and multiple small areas of mineralization (Figure 5). In some areas away from the main focus of inflammation, muscle fibers were separated by edema fluid and lesser numbers of macrophages. The IHC-stained section of this area revealed foci of PrP<sup>Sc</sup> within the macrophages (Figure 6).

Except for the tongue of sheep 3511, the IHC-stained sections of non-CNS and nonlymphoid tissues, including striated muscle (heart, diaphragm, and masseter muscle), did not reveal PrP<sup>Sc</sup>. The adrenal medulla of sheep 3514 revealed PrP<sup>Sc</sup> in the cytoplasm of multiple endocrine cells in a focally restricted area.

In the WB analysis with monoclonal antibody P4, brainstem samples from all the sheep except the negative-control lamb were positive for PrP<sup>Sc</sup>, showing the typical profile of 3 bands of proteinase-K-resistant isoforms of PrP<sup>Sc</sup> (Figure 7), which represent the diglycosylated, monoglycosylated, and unglycosylated polypeptides. The molecular pattern of the 3 isoforms in the samples from the animals inoculated by the IC route was similar to the pattern for the animals inoculated by the IL route.

In this study, the localization of PrP<sup>Sc</sup> deposits in brain, lymphoid tissues, and retina in the scrapie-positive sheep was similar to that previously seen in sheep with scrapie after inoculation by the oral or IC route (2). Labeling of variable intensity was seen in various sections of the brain. The labeling was predominantly either particulate and diffuse or multifocal and extensive but also appeared as scattered small aggregates of consolidated plaques. Labeling



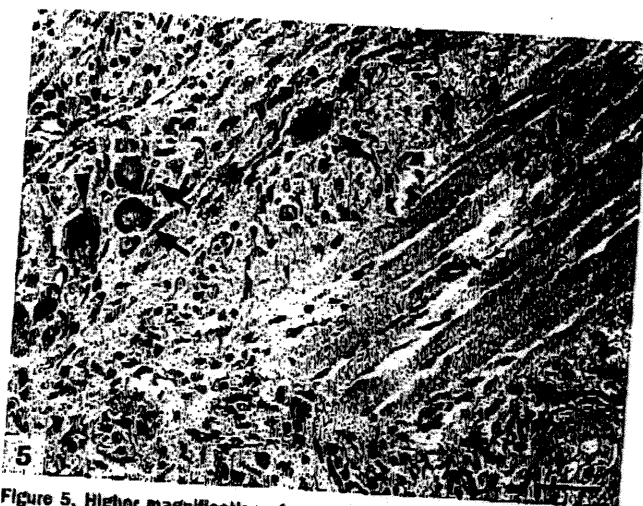


Figure 5. Higher magnification of area demarcated in Figure 4, showing multinucleated giant cells (arrows) and isolated mineralized muscle fibers (arrowhead). H-E. Bar — 40  $\mu$ m.



Figure 6. Tongue of sheep 3511, showing focal area of dense PrP<sup>Sc</sup>-positive material (red) in the area of inflammation and, in the inset, within the epithelioid macrophages. Immunohistochemical phosphatase and hematoxylin. Bar — 40  $\mu$ m.

predominated in the grey matter neuropil as perineuronal accumulations and in the perikaryon. Staining in the white matter was markedly less intense and appeared as scattered aggregates of particles. Labeling of the inner and outer plexiform layers of the retina was characterized by diffuse coalescing particles forming roughly uniform sheets. In IHC-positive lymphoid organs, PrP<sup>Sc</sup> labeling was either particulate or appeared as small aggregates of particles. Sparse punctate labeling of cells in the paracortex was noted in some sections.

Labeling of the tongue with PrP<sup>Sc</sup> was documented in 7 of 10 sheep with naturally occurring scrapie in European breeds of sheep (6). However, in the present study, except for the sheep euthanized 9 d after inoculation, which had IHC staining of the tongue, PrP<sup>Sc</sup> labeling was not observed in striated muscles of the scrapie-affected animals. This observation is in accordance with previous findings in 20 animals (cattle, sheep, elk, and raccoons) that had a TSE after experimental inoculation: PrP<sup>Sc</sup> was found by IHC examination in the CNS but not in striated muscle tissues (7). Recent investigations with an enriched WB technique (8,9) have enabled the detection of PrP<sup>Sc</sup> in the tongues of some sheep and elk experimentally infected with the agents of scrapie and chronic wasting disease (CWD), respectively (Richard Beasen, Montana State University, Bozeman, Montana; personal communication, 2006). This technique, however, failed to detect PrP<sup>Sc</sup> in tongues of cattle in which a TSE developed after inoculation with the agent of CWD or that of transmissible mink encephalopathy (TME) (Richard Beasen, Montana State University; personal communication, 2006). The contrast in IHC results between the present study and the European study (6) could be due to differences between scrapie strains, breeds of sheep, natural and experimental inoculation, or the IHC procedure.

Inoculation route apparently did not influence the molecular phenotype of the PrP<sup>Sc</sup> observed in the CNS tissues in our study. Alternative routes of infection and spread of prion disease have been suggested as being responsible for different disease pheno-

types, as in cattle with typical and atypical bovine spongiform encephalopathy (9).

In general, the IC route of inoculation is considered to be the fastest means of transmitting TSE agents to susceptible hosts. In a study of sheep injected by the IC route with a different US scrapie inoculum (no. 13-7), the average incubation time was 18.8 mo (2); in the present study, with injection by this route of a different US scrapie inoculum (no. X124), the mean time to development of advanced clinical signs of scrapie was 17.6 mo. The sheep in both studies had clinical signs of scrapie, lesions of spongiform encephalopathy, and PrP<sup>Sc</sup> deposits in their tissues. Although sheep 3504 in our study had a significantly longer survival than the other sheep inoculated by the IC route, no difference in survival time was noted between the IL and IC groups. Recently, hamsters in which TME agent was inoculated into the tongue and 4 other non-neuronal anatomic sites were found to have PrP<sup>Sc</sup> in submandibular lymph nodes and the hypoglossal nucleus in the brain within 2 wk after inoculation (10).

The IC route of inoculation is an invasive surgical procedure that involves the use of light anesthesia and carries the risks of intracranial hematoma, CNS infection, and complications of anesthesia. Aside from being safer, the IL method may mimic the natural route of infection for prion diseases. Development of an inoculation method that has an incubation time comparable to that of the IC method and is safer for the recipient animals is desirable. Although the numbers of animals used in this study were small, the results of the IL method were comparable to those of the IC method, and the former can be performed without general anesthesia. However, before the IL method of inoculation can be recommended for general use in TSE studies, similar studies need to be done with other TSE agents and need to involve larger numbers of susceptible hosts to validate the efficacy of the IL inoculation method.

Results of this study show that the transmission of scrapie via the IL route is possible. In comparison, oral inoculation required an average survival time of 32 mo and resulted in a lower attack rate (2). In another study (9), the IC route required a smaller volume of



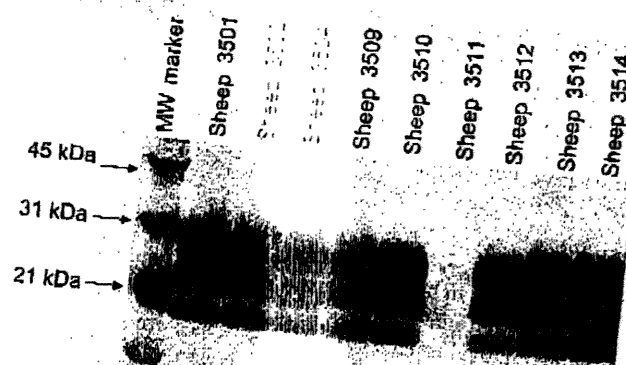


Figure 7. Western blot analysis of brainstem material from sheep inoculated with scrapie agent X124 by the intralingual or the intracerebral route. The immunoblot was developed with the use of monoclonal antibody P4. MW — molecular weight.

inoculum than the oral route (1 mL versus 30 mL of a 10% brain suspension), yet had a higher attack rate (100% versus 56%). It therefore appears that under field conditions the oral cavity in general, and the tongue in particular, may serve as a portal of entry for prions to gain access to the host's  $Pr^{Psc}$ -susceptible cell population.

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names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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2 A Comparative Study Utilizing Immunohistochemistry on Lymphoid Tissue of the Third  
3 Eyelid and Rectal Mucosa for the Diagnosis of Scrapie in Sheep  
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**Objectives:** To compare scrapie immunohistochemistry done on biopsies of rectal lymphoid follicular tissue with that of the third eyelid of scrapie-exposed sheep to determine which is the better tissue for PrP<sup>sc</sup> diagnosis.

**Design:** Two hundred-ten scrapie-exposed sheep were tested for three years.

**Animals:** Sheep from infected flocks were identified, genotyped and bred. Beginning at 12 months of age, sheep had eyelid and rectal lymphoid biopsies taken annually.

**Procedures:** The tissues were biopsied, fixed in formalin, sectioned for immunohistochemistry and stained for prion protein. Brain, tonsils, retropharyngeal, mesenteric, mandibular lymph nodes and remaining tissue from third eyelids and rectum were sampled from animals that died and tested also.

**Results:** Eight-three animals were positive based on results from eyelid, rectal follicular tissue or from necropsy results. Sixty-eight percent of the positive animals, tested positive on the eyelid biopsy, 72.3% tested positive on the rectal biopsy. Forty-two animals tested positive on both biopsies. The youngest positive sheep were codon 136/171 AVQQ and VVQQ. The average age of animals having "no follicles" in one or more biopsies was 4.2 years. The oldest positive animals were positive on the rectal but negative on eyelid biopsy. Nine negative sheep on biopsy were found to be positive on necropsy.

**Conclusions and Clinical Relevance:** The rectal biopsy seems to be slightly superior to the third eyelid. However, sampling both sites appeared to be far more advantageous. Utilization of the rectal site only would have missed 28% of the positive animals.

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Scrapie appears to be the result of accumulating post-translationally **modified cellular protein (PrP<sup>SC</sup>)**, a reliable indicator of the presence of the infective scrapie agent in sheep<sup>1</sup>. The PrP<sup>SC</sup> apparently acts as a template for the conversion of **normal cellular protein (PrP<sup>C</sup>)** to PrP<sup>SC</sup> resulting in a conformational change whereby its alpha-helical content diminishes and the beta-sheet content increases. These structural differences change its chemical properties and possibly its pathogenesis.

The source of scrapie is believed to be infected birthing fluids and placenta; the route of naturally occurring infection is orally either by the lamb during or after birth or other adults licking, drinking or eating the infected fluids and placenta<sup>2</sup>. Access into the body is probably via ileal villous lacteals and sub mucosal lymphatics<sup>3</sup>. From there it can be demonstrated in dendritic-like cells in the draining lymph nodes up to 24 hrs post challenge. Replication of infection, demonstrated by accumulation of PrP<sup>SC</sup> in Peyer's patches has been detected at 30 days in inoculated sheep<sup>3</sup>. In some studies the accumulation of PrP<sup>SC</sup> in the tissues of the lymphoreticular system has been shown to facilitate neuro-invasion<sup>4</sup>. Once infection replicates in the lymphoid nodules of the gut-associated lymphoid tissue, dissemination to the other lymphoreticular and nervous tissues can occur via blood and/or lymph<sup>5</sup>. The eventual outcome is a progressive, debilitating, neurological illness, which is always fatal.

The presence of PrP<sup>SC</sup> in lymph tissue of the body generally precedes that in the brain. Using **PrP immunohistochemistry (IHC)**, PrP<sup>SC</sup> can be detected in lymphoid tissue such as that of the spleen, tonsil, retropharyngeal, mesenteric and prescapular lymph nodes of affected animals prior to accumulation in the CNS. This progression of PrP<sup>SC</sup> infectivity has led to sampling of easily accessible superficial lymphoid tissue for



preclinical testing for scrapie<sup>6,7</sup>. Lymphoid follicles found in tissues of the **third eyelid (EL)**, easily sampled in the live, non-anesthetized animal, for example, have been adopted as a live animal test for the scrapie eradication program<sup>8</sup>.

However, the technique is hampered by the lack of an adequate number of follicles for PrP<sup>SC</sup> examination in a significant number (as high as 40%-60%),<sup>9</sup> in some groups of adult sheep. The mandibular lymph node is another possibility, almost always having follicles, but requires simple surgery, making it a less convenient sample. It was recently reported that PrP<sup>SC</sup> can be found in scrapie-positive sheep within **lymphoid follicles found at the muco-cutaneous border of the rectum (RF)**<sup>10</sup>.

The following report summarizes data gathered from 210 sheep naturally exposed to scrapie, comparing IHC results from EL follicles and RF for the presence of PrP<sup>SC</sup> in biopsy (live) as well as necropsy samples where the results could be compared with the presence of PrP<sup>SC</sup> in the brain and other lymphoid tissue.

## **Materials and Methods:**

Animals: Between 1996 and 1999, flocks in the State of Idaho diagnosed with scrapie were subjected to depopulation and donated to the University of Idaho, Caine Veterinary Teaching Center for scrapie research. Since then, sheep numbers have been kept at about 200 head by selective euthanasia. Approximately 35 head of 171 codon **glutamine (Q), arginine (R)** genotype have been maintained and the rest are 171 QQ. Codon 136 **valine (V), alanine (A)**, and AV genotypes are present in the flock. All sheep in this study were codon 154 RR. For this report, genotype is expressed by recording codon 136 first, followed by codon 171. Each sheep is uniquely identified and



individual records are kept on each animal. The ewes are bred each year to insure a high degree of exposure and continued transmission of scrapie in the flock.

The animals are kept in outside dry lot pens with fence-line feeders and shelter from inclement weather. Their base diet is alfalfa hay. Grain is fed during pregnancy and lactation. Heated, automatic waterers provide water ad lib. A trace mineral salt with selenium for sheep is provided. Technicians check the animals twice a day and record any problems or abnormal behavior. Sick animals are separated into a sick pen for special care. Beginning at approximately 12 months of age, sheep have EL biopsies taken annually and since 2005, rectal biopsies have been added to the protocol. Facilities and care meet Federal ACU specifications.

Biopsy protocol: For EL biopsy, the O'Rourke and Parrish technique was followed<sup>11</sup> with the exception that xylazine (0.2 mg/lb) was given IV for sedation and analgesia.

Rectal tissue just inside the muco-cutaneous border is picked up by a pair of forceps and snipped off using scissors as previously described<sup>10</sup>. The piece of tissue, approximately 0.5 x 1.0 cm in size, is placed in a sponge-containing cassette and dropped into formalin. Instruments are soaked in 2.5 N NaOH overnight, rinsed, autoclaved and reused at a later date.

Necropsy protocol: Animals affected with scrapie were euthanized when they no longer were able to keep themselves upright. Animals with other problems were also euthanized when they became unresponsive to treatment. Selective euthanasia was done on sheep older than 18 months, chosen randomly, that had negative tests on EL and RF biopsies. Necropsies were done immediately after euthanasia. If gross abnormalities



were present, samples of affected parts are taken for bacteriological examination and/or placed into formalin for microscopic examination. Brain, tonsil, retropharyngeal lymph node, mandibular lymph node, EL lymphoid tissue (if still present), RF, and intestinal lymph node were sampled and placed into 10% buffered formalin for IHC.

Immunohistochemistry test:

Tissues were grossed and put into cassettes. The tissues in the cassettes were washed in running tap water for 15 min and then treated with a bath of 95% formic acid for one hour. A two to three hour rinse in running tap water followed before processing for sectioning. The tissues were cut at 4 microns. The IHC stain was done on a Ventana Benchmark LT<sup>a</sup>, and all reagents used were Ventana products. The detection kit used was the UltraView Red<sup>b</sup> with hematoxylin as a counter stain followed by a bluing reagent. The incubation time for the anti-prion antibody 99/97.6.1 was 24 minutes. Five follicles were required on a biopsy to be read as “negative”, otherwise it was recorded as “**no follicles**” (NF). Suspicious results were recorded when deposits were lightly dispersed and scarce. For this study, however, suspicious results were considered positive.

**Results:**

A total of 210 animals had both EL and RF tissue tested for PrP<sup>SC</sup> by biopsy.

Twenty-four of those have died and had tissues tested at necropsy.

Seventy-four animals were considered positive by biopsy (Tables 1 and 2). Nine additional animals with both negative EL and RF tissue were found to have other positive



tissues on necropsy, i.e. brain, tonsils, retropharyngeal and/or other lymph nodes, making a total of 83 positive animals. One of the nine had PrP<sup>SC</sup> only in the brain.

Thirty-five (14 EL and 21 RF, 6.7% and 10.0%, respectively) had one or both biopsies recorded as NF. These animals tended to be older (Table 3); those that had NF recorded for the RF averaged 4.1 years, those for EL averaged 3.1 years. Twenty-four of the 35 were positive on the opposing lymph node. Five of the remaining 11 animals in which the opposing tissue was negative have died and were confirmed as negative on necropsy tissues.

One hundred eleven of the tested animals (52.9%) were considered negative, having agreement on the two biopsies. However, 9 of those (4.3%) were found to be positive after necropsy. Ages of these 9 animals ranged from 1.1 to 7.8 years of age. One of the 9 had PrP<sup>SC</sup> only in the brain. Those that had both positive EL and RF tissues on necropsy averaged 1.7 years.

Of the 83 positives, 56 (67.5%) had positive EL of which 8 were negative on RF and 5 were NF. Fifty-nine (71.1%) had positive RF of which 42 (50.6%) had positive EL and there were 16 positive RF samples for which the EL was negative. So far, 10 of those deemed to be positive on the live tests have died and been confirmed on necropsy.

The predominant genotype (Table 4) of the negative scrapie-exposed sheep was AAQQ (81.1%) but then, it made up 43% of the entire group of 210. The predominant genotype of the positive sheep was AVQQ (37.3%) while it made up only 29.5% of the entire group. The VVQQ animals made up only 11% of the group with a total of 23 animals; 22 were considered positive on biopsy. The remaining VVQQ animal was negative in EL and RF, but was positive upon necropsy; making 100% of the group



positive. It died at 2 yrs of age with clinical symptoms of scrapie. Oddly, the brain tested negative for PrP<sup>SC</sup> while lymphoid samples were positive. Nineteen of the VVQQ animals were positive for PrP<sup>SC</sup> in both biopsy tissues. Twelve of the 23 have died and have been confirmed by necropsy results as well.

The AAQR animals in the flock totaled 19; 10.5% of the group tested positive. One had PrP<sup>SC</sup> in both biopsy tissues, one was positive in the EL and had NF in the RF. One animal was 4.3 yrs while the other lost its original tag and thus the age was unknown. There were no positive biopsies in the 15 AVQR sheep.

#### **Discussion:**

Actually, for this study the number of NF for EL (6.7%) was less than we have encountered in the past<sup>12</sup>. However, only 50.6% of the animals deemed positive tested positive in both tests. Interestingly, these animals that tested positive in both tests averaged between 1.1 and 3.0 years.

There were 17 RF only positive and 14 EL only positive samples. Since just 3 of these have died, those results are not yet confirmed. However, so far those that have died with a positive test in one or more biopsy sites have been confirmed. It appears that the follicles of the eyelid may be slightly less likely than the RFs to collect PrP<sup>SC</sup>. Genotype appears to affect susceptibility. Only 20.5% of the positive animals were AAQQ though they made up the majority of the flock (42.9%). On the other hand, 37.3% of the total positive animals were AVQQ even though this genotype totaled only 62 animals. Half (50.0%) of this genotype group was positive. Further, all 23 of the 23 VVQQ genotype were positive. Considering that these were only a small percentage (11.0%) of the flock, this confirms the high susceptibility of this genotype to scrapie as reported elsewhere<sup>13</sup>--



183 particularly when exposed to a V-dependant strain of scrapie<sup>14</sup>. Noteworthy is the  
184 finding that two of 19 (10.5%) AAQR sheep, considered to be quite resistant, were also  
185 positive whereas none of the 15 AVQR sheep (considered to be quite susceptible to the  
186 V-dependent strain of scrapie<sup>8,14</sup>) were positive at time of testing. Previous results have  
187 suggested that it is likely that there is a V-dependent or some different strain in addition  
188 to the usual U.S. strain of scrapie agent in this flock.<sup>15</sup> Thus the pattern of genotypes  
189 falling prey to scrapie in this flock is probably much different than that seen in flocks  
190 affected by just one of the strains of scrapie more commonly found in the general  
191 population of U.S. sheep. The relatively high percentage of AAQR sheep testing positive  
192 may be a result of the high exposure received by these sheep but doesn't explain apparent  
193 resistance of the AVQQ sheep.

194         Nine of the 83 positive animals showed no PrP<sup>SC</sup> in either peripheral biopsy but  
195 were positive in tissues other than EL or RF or in just the brain at necropsy. Four of the  
196 nine animals were genotype AAQQ and 5 were AVQQ, thus accumulation of PrP<sup>SC</sup> in  
197 peripheral lymph tissue does not seem to be affected by genotype nor is the lack PrP<sup>SC</sup> in  
198 lymphoid tissue unique to the just one breed of sheep<sup>16</sup>. Since only 14 designated  
199 negative animals have died so far and been necropsied, it seems very likely that the false  
200 negative group will be much higher.

201         The VVQQ animal testing as negative in EL and RF tissue, then found to be  
202 positive after necropsy, died at 2 yrs of age with clinical symptoms of scrapie is  
203 particularly fascinating. Although all lymphoid tissues tested positive, the brain tested  
204 negative for PrP<sup>SC</sup> in spite of the animal dying as a result of its clinical signs. This  
205 animal is not totally unique. In a previous report, genotypic AVQQ animals dying with



clinical signs of scrapie had light and scarce deposition of PrPr<sup>SC</sup> in brain tissue<sup>15</sup> and there is also evidence of this in other species<sup>17</sup>. Certainly, the IHC evidence was not enough to explain the signs exhibited by the animals. These situations certainly increase the evidence that accumulation of PrP<sup>SC</sup> in tissues is the result of some other etiologically agent and not itself the etiologic agent of the disease and lends credence to other reports.<sup>18,19,20,21</sup>

Genotype, as reported elsewhere,<sup>13</sup> also appeared to be associated with incubation time, particularly when related to the 136V genotype<sup>21</sup>. Seventeen AAQQ animals that tested positive in one or both EL and RF were an average of 3.1 years of age; 31 AVQQ animals averaged 1.6 years of age and the 23 VVQQ averaged 1.5 years of age, when identified as positive. Many of the AVQQ and the VVQQ animals tested positive on their first sampling at approximately one year of age. For our purposes, it is important that we identify our positive animals early and we believe it would be useful to start testing these two genotypes earlier than one year of age.

Follicular tissue tends to diminish with age. The EL tests start losing follicles earlier than RF samples (3.1 yrs vs. 4.1 yrs). There is some suggestion that the amount of lymphoid tissue ringing the anus is less at the dorsal and ventral location. Obviously experience improves the results for RF sampling as well as for the EL. Never the less, it is interesting that NF was greater for RF than EL and the problem remains a major drawback of both procedures.

Since we will be testing the negative animals again and again, we only take one sample from a site at a time, leaving remaining samples for later testing. Any animal with a suspicious test is considered positive and kept in the flock but tested again the



229 following year to hopefully confirm the suspicious diagnosis. Testing necropsy samples  
230 gives the final diagnosis. As incubation time can be as long as 5 plus years, many of our  
231 animals, particularly those with AAQQ and AAQR genotypes may not be confirmed for a  
232 couple of years.

233         Since 50.6% of our positive animals were positive on both samples, it does appear  
234 that taking both biopsy samples is likely to be far more rewarding than sampling from  
235 just one site. Taking multiple samples from the rectum may also have improved the  
236 sensitivity of the rectal biopsy.

237         Actually, if presented with a client's animal showing signs suggestive of scrapie,  
238 a practicing veterinarian may wish to take several rectal biopsies, an eyelid sample and a  
239 biopsy of the mandibular lymph node to be tested. In this instance, increasing the  
240 likelihood of an animal actually not having scrapie is important especially if the animal is  
241 valuable and the owner wishes to attempt treating the animal.

242         Having a simple, low-cost, relatively rapid, dependable and sensitive live animal  
243 test is important for us and very important for scrapie eradication in all sheep raising  
244 countries. Thus, many researchers are working diligently on a blood test. Certainly  
245 blood would be a far more convenient sample, but prions in the blood have been elusive.

246         Recently, however, a test has been reported for the detection of PrP<sup>SC</sup> in the blood  
247 of infected but asymptomatic animals<sup>23</sup>. This procedure combines two very sensitive  
248 assays, an *in vitro* amplification step with a novel aggregation-specific ELISA and a  
249 fluorescent amplification catalyzed by T7 RNA polymerase technique. This procedure  
250 can detect PrP<sup>SC</sup> at femtomolar levels.



251           This is an exciting development but tests on blood from sheep with clinical signs  
252 have not yet been reported. Further, it is not likely that this test will be simple,  
253 inexpensive or rapid, at least not in the near future. Thus lymphoid biopsy and IHC is  
254 likely to be our best testing procedure for some time to come.

255 <sup>a</sup>Ventana Medical Systems, Inc. 1910 E. Innovation Park Dr. Tucson, AZ 85755

256 <sup>b</sup>Ultra Red Veiw Kit: Ventana Medical Systems, Inc. 1910 E. Innovation Park Dr.  
257 Tucson, AZ 85755

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**Table 1.** Comparison of Third Eyelid and Rectal Lymphoid Tissue Biopsies for Presence of PrP<sup>SC</sup> in 83 Animals Diagnosed as Positive for Scrapie

	<b>Rectal positive</b>	<b>Rectal negative</b>	<b>No rectal follicles</b>	<b>Total</b>
<b>Eye positive</b>	42 (50.6%)	8 (9.6%)	6 (7.2%)	56 (67.5%)
<b>Eye negative</b>	16 (19.3%)	9*(10.8%)	NA	25 (30.1%)
<b>No eye follicles</b>	2 (2.4%)	NA	NA	2 (2.4%)
<b>Total</b>	60 (72.3%)	17 (20.5%)	6 (7.2%)	83 (100%)

\*Animals diagnosed as positive on tissues besides EL and RF on necropsy.

**Table 2.** Comparison of Third Eyelid and Rectal Lymphoid Tissue Biopsies for Presence of PrP<sup>SC</sup> by Immunohistochemistry Staining in 210 Naturally Exposed Animals

	<b>Rectal positive</b>	<b>Rectal negative</b>	<b>No rectal follicles</b>	<b>Total</b>
<b>Eyelid positive</b>	42 (19.8%)	8 (3.8%)	5 (2.4%)	55 (26.2%)
<b>Eyelid negative</b>	16 (7.5%)	112*(52.8%)	13 (6.1%)	141 (66.5%)
<b>No eye follicles</b>	1 (0.4%)	1 (5.2%)	2 (0.9%)	14 (6.7%)
<b>Total</b>	59 (28.1%)	131 (61.8%)	20 (9.5%)	210 (100%)

\*Nine of these animals were found to be positive on necropsy



**Table 3.** Average and Range of Age of Sheep in Years Showing Presence of PrP<sup>SC</sup>

within Lymphoid Follicles (and Those with No Follicles) of the Third Eyelid and Rectal

Mucosal Tissue Using Immunohistochemistry Staining

<b>Tissue Results</b>	<b>Rectal positive</b>	<b>Rectal negative</b>	<b>No rectal follicles</b>
<b>Eye positive</b>	1.7 (1.1-3.0)	1.8 (1.3-2.3)	2.6 (1.1-4.4)
<b>Eye negative</b>	2.3 (1.1-5.6)	2.9 (1.1-7.8)	4.1 (1.1-5.8)
<b>No eyelid follicles</b>	1.6 (1.1-2.2)	3.1 (1.1-4.5)	4.2 (2.1-6.0)



**Table 4.** Comparison of Genotypes, Age Range and Average Age of 83 Scrapie Positive and 127 Scrapie Negative Animals Diagnosed using Immunohistochemistry Staining on Third Eyelid and Rectal Lymphoid Biopsy and Necropsy Samples.\*

<b>Genotype</b> Tissue Results	<b>AAQQ</b>	<b>AAQR</b>	<b>AVQQ</b>	<b>VVQQ</b>	<b>AVQR</b>
EL+, RF+	7	1	15	19	0
Age range, years	1.75-2.7	Unk	1.1-3.0	1.1-2.3	
age avg, years	2.1		1.9	1.5	
EL+, RF NF	1	1	2	1	0
Age range, years			1.1-1.8		
Age avg, years	4.4	4.3	1.5	1.8	
EL+, RF neg	4	0	3	1	0
Age range, years	1.5-5.3		1.8-2.3		
Age avg, years	2.7		2.0	1.3	
EL NF, RF +	0	0	1	0	0
Age range, years					
Age avg, years			2.2		
EL neg, RF+	5	0	10	1	0
Age range, years	4.2-5.6		1.1-2.0		
Age avg, years	4.9		1.2	1.1	
EL neg, RF neg	56	15	26	1	13
Age range, years	1.5-7.8	3.3-7.8	0.8-6.8		1.1-6.8
Age avg, years	2.8	4.3	1.8	1.8	2.9
EL NF, RF neg	8	0	2	0	1
Age range, years	2.3-5.4		1.1-1.8		
Age avg, years	3.9		1.4		1.3
EL neg, RF NF	8	2	2	0	1
Age range, years	3.3-5.8	3.3-4.3	1.1-1.3		
Age avg, years	4.3	3.8	1.2		2.0
EL NF, RF NF	1	0	1	0	0
Age range, years					
Age avg, years	4.3		2.1		
Total negative	73	17	31	1	15
Age range, years					
Age avg, years					
Total positive	17	2	31	22	0
Age range, years					
Age avg, years					
Total	90	19	62	23	15
Age range, years					
Age avg, years					

\*=the genotype of one sheep was not available



**University of Idaho  
Animal Care and Use Committee**

**Date:** Tuesday, May 09, 2006  
**To:** Marie Bulgin  
**From:** University of Idaho  
**Re:** Protocol 2006-50  
Efficient and rapid development of transgenic hamster models of TSE's using a radical new technology

Your animal care and use protocol for the project shown above was reviewed by the University of Idaho on Tuesday, May 09, 2006.

This protocol was originally submitted for review on: Friday, March 10, 2006  
The original approval date for this protocol is: Tuesday, May 09, 2006  
This approval will remain in affect until: Wednesday, May 09, 2007  
The protocol may be continued by annual updates until: Saturday, May 09, 2009

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.



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IACUC Representative



**Brad Williams, DVM**

---

**From:** Sharon Sorensen [sorensen@uidaho.edu]  
**Sent:** Monday, May 08, 2006 12:37 PM  
**To:** Brad Williams, DVM  
**Subject:** RE: protocol 2006-50

Thank you for reminding me that Jason is on the protocol. He is leaving us on June 16, but will do the training module anyway, hopefully today. I told him it was important. I have the medical form here for him to sign also.

Sharon



## Brad Williams, DVM

---

**From:** Brad Williams, DVM [bradw@uidaho.edu]  
**Sent:** Monday, May 08, 2006 10:29 AM  
**To:** 'sorensen@uidaho.edu'  
**Cc:** DVM Marie Bulgin (mbulgin@uidaho.edu); 'Linda DuBose'  
**Subject:** RE: protocol 2006-50

Sharon,

> 1. The flunixin meglamine concentration is 50 mg/ml

This is why there was confusion. I've never seen any but 100 mg/ml. Ok.

> 5. All personnel have completed the online training module and exam  
> and have signed a participation or declination form for the Animal  
> Workers Medical Surveillance Program.

Jason Down has not completed the online training module and exam and I have no AWMSP forms for him. Once we get that cleared up the approval can go forwards. For the moment, the Office of Sponsored Programs is not cutting off the budget supporting this project. Not sure how much longer I can hold them off. Any way to get Jason to do these today or tomorrow?

Brad



**Brad Williams, DVM**

---

**From:** Sharon Sorensen [sorensen@uidaho.edu]  
**Sent:** Monday, May 08, 2006 10:07 AM  
**To:** bradw@uidaho.edu  
**Cc:** mbulgin@uidaho.edu  
**Subject:** protocol 2006-50

Brad,

In response to your questions:

1. The flunixin meglumine concentration is 50 mg/ml, and the dose is 1-2 ml/100 lbs, given twice, 6-8 hrs apart. The reason I used a range was at your suggestion and some of these sheep are 300 pounds, which requires a dose of 6 ml.
2. The ketamine concentration is 100 mg/ml, with a dose of 5-15 mg/lb.
3. Metabolism crates have expanded metal floors to allow the urine to run through and be collected via a large funnel device under the floor.
4. The surgeon is Dr. Marie Bulgin, DVM.
5. All personnel have completed the online training module and exam and have signed a participation or declination form for the Animal Workers Medical Surveillance Program.

Dr. McKelvey was here last week about a different matter, but has inspected us before.

I hope this allows us to be approved.

Thank you for your help with this protocol.

Sharon



**University of Idaho  
Animal Care and Use Committee**

**Date:** Friday, April 28, 2006  
**To:** Marie Bulgin  
**From:** University of Idaho  
**Re:** Protocol 2006-50  
Efficient and rapid development of transgenic hamster models of TSE's using a radical new technology

Your animal care and use protocol for the project shown above was reviewed by the University of Idaho on Friday, April 28, 2006. The committee requests further information or clarification of the following points.

1. The flunixin meglumine dose is listed as 1.5-6 ml. Typical dosage is 1 ml/100 lb body weight. A 6 ml single dose may likely result in GI ulcers and/or renal disease. What is the dosage in mg/lb or mg/kg and for how long will it be administered?
2. The ketamine dose needs to be specified in a mg/kg or mg/lb format.
3. Sheep will be maintained in metabolism crates for urine collection, but how will the urine itself be collected?
4. The surgery form does not identify who the surgeon is or their qualifications. Who will be performing the surgical procedures and what are his/her qualifications for doing so?
5. All personnel must complete the online training module and exam (<http://www.uro.uidaho.edu/acuc/training>) and participation or declination forms for the Animal Workers Medical Surveillance Program (<http://www.uro.uidaho.edu/acuc/ehs>).

**Comments**

Because this project is being conducted to supply tissues for use in human prion research, it qualifies as an animal model of human disease and is therefore subject to USDA oversight. The sheep and sheep housing used to support this project should be made available to the USDA inspector when he/she comes.

**Response to this letter does not constitute approval to begin the proposed study.**



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IACUC Representative



**University of Idaho  
Animal Care and Use Committee**

**Date:** Thursday, March 22, 2007  
**To:** Marie Bulgin  
**From:** University of Idaho  
**Re:** Protocol 2006-50  
Efficient and rapid development of transgenic hamster models of TSE's using a radical new technology

was reviewed by the University of Idaho on Thursday, March 22, 2007.

This protocol was originally submitted for review on: Friday, March 10, 2006  
The original approval date for this protocol is: Tuesday, May 09, 2006  
This approval will remain in affect until: Wednesday, May 09, 2007  
The protocol may be continued by annual updates until: Saturday, May 09, 2009

**Comments**

Your request for an additional euthanasia method using intravenous pentobarbital overdose is approved.

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.



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IACUC Representative



Biohazard \_\_ yes X no

Radioisotope \_\_ yes X no

Chemical Carcinogen \_\_ yes X no

Agent \_\_\_\_\_

Agent \_\_\_\_\_

Agent \_\_\_\_\_

ACUC USE ONLY	Protocol Number	2006-50	Expiration Date	
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1. Investigator: Dr. Marie Bulgin Dept: AVS/CVTC Phones: 208 454 8657

Co-investigator: Sharon Melson Dept: AVS/CVTC Phones: 208 454 8657

Technician: Trisha Walker, Brian Meyer Dept: AVS/CVTC Phones: 208 454 8657

Student: \_\_\_\_\_ Dept: \_\_\_\_\_ Phones: \_\_\_\_\_

2. Species (common names/Estimated numbers per year) sheep, 50

3. Location of animal housing (bldg & rm) Caine Veterinary Teaching Center, Caldwell Idaho

4. Proposed duration of project 2 years 5. Funded DOD Contract #DAMD17-03-1-0746, subcontract UI

6. Project Title or Course Name and Number: Efficient and rapid development of transgenic hamster models of TSE's using a radical new technology

7. Abstract of Research/Teaching Plan for the Information of Animal Care Staff

In the space provided, give a brief layman's description of the procedures involving animals.

In order to determine when the disease onset occurs after exposure, but before clinical symptoms show up, third eyelid and rectal mucosa samples will be collected twice a year for immunohistochemistry testing for scrapie. Monthly bleeding of some animals will be done for TSE analysis, plus, urine, tissue and blood sampling at necropsy. Urine samples may also be collected from live animals using metabolic cages at the request of the researchers on an as needed basis.

8. Special Requirements for maintaining animals: X no \_\_ yes. If yes, indicate your requirements below. If you have no special requirements, animals will be maintained according to the standard operating procedure of the vivarium.

a. Temperature range: (°F)\_\_\_\_; humidity (%): \_\_\_\_; light cycle:

b. Caging: type corral; size: \_\_\_\_; filter tops? \_\_; cage changes/wk:

c. Bedding/litter: type dirt, straw; autoclaved? \_\_; bedding changes/wk:

d. Type of water (ie sterile, deionized, acidified, tap) tap

e. Diet and feeding requirements: Special diet? Alfalfa hay

If other than ad lib feed & water, state amounts:

f. Other Special Instructions for Animal Care Staff: Report any abnormal behavior or gait problems.

9. Check all applicable boxes. If more than one box checked per heading, number in order to be accomplished.

Instruction for sick animals

Instructions for Dead Animals

Pest Control

X Call Investigator

X Call Investigator

X None



☐ Clinician to treat

☐ Necropsy

☐ Pest strip

☐ Terminate

☐ Bag for disposal

☐ Pyrethrin ☐ Other (specify)





Please type, handwritten forms will be returned

2002 Version

Name of Principal Investigator <b>Marie S. Bulgin</b>	Department d <b>AVS</b>	Phone No. <b>208-454-8657</b>
Co-Investigator <b>Sharon Melson</b>	OFFICE USE:  Date Rec'd. IACUC #	
Name of Funding Agency <b>DOD Contract #DAMD17-03-1-0746, subcontract UI</b>	Approved: Date:	
Title of Proposal <u><b>Efficient and rapid development of transgenic hamster models of TSE's using a radical new technology</b></u>		

PROPOSED PERIOD: 9/1/2005 TO 8/31/2007

Is confidential information contained in the project? Yes X No

If yes, please identify by circling item number or highlighting

Is this a renewal or reapplication of a previously approved project? X Yes No

If yes, list last approval IACUC Number. 2003-53

Are there any changes to the previously approved project listed above? X Yes No

1. ANIMAL PROCUREMENT AND CARE (Attach additional sheets if required)

Species (Breed/Strain) of Animals	Source of Animals	Approx. Total No. of Animals to be Used			Daily Per Diem	Total Days of Care	Total Cost of Care
		C	D	E			
Blackface ovine	Donation or born in flock		50		1.00	365/yr	\$356/hd/yr



**\*For type classification, see instruction sheet enclosed. Type E projects require written justification statement for review.**



2. **PURPOSE OF STUDY** (In non-scientific terms, describe the overall purpose of the study, the rationale for using animals, and the relevance of the project to human/animal health or biology. For each portion of the study using animals, describe the species, sex, age, and number used. Be concise.)

To rapidly develop transgenic hamsters models of TSE. This sheep flock of 50 animals will provide biological test materials from genetically characterized sheep. Transgenic rodents (rodents given sheep genes) will be inoculated with various extracts of tissues and blood from this flock to check for infectivity and to determine if the scrapie prion can be quantified. The transgenic hamsters reside in the research laboratories of Dr. Robert Rohwer in Baltimore Maryland and he is responsible for the rodent inoculation. A great deal of research has been done in the model previously, thus his choice to continue using hamsters.

A by-product of the TSE study in hamsters is the study of the disease in the natural host (sheep). the sheep provides larger sample sizes and the natural exposure guarantees the "real" agent and not one changed by laboratory manipulation. Plus the accumulation of positive TSE material to be used for various positive test controls.



3. ANIMAL NUMBERS AND HOUSING AREA

☐ Laboratory Animal Resources Facility (LARF)

☐ Holm Center Vivarium

☐ Dairy Center

☐ Beef Center

☐ Sheep Center

☐ USDA Hemoparasitic Barn

☐ NERL Facilities

☐ Intensive Animal Research Barn

☐ Aquaculture Research Institute

☐ Aquaculture Laboratory

☐ Fish & Wildlife Fish Rooms

☐ Idaho Wildlife Health Laboratory

☒ Caine Veterinary Teaching & Research Center

☐ Caldwell R&E Center

☐ Hagerman Fish Culture & Experiment Station

☐ Other (please describe)

Has the proposed study been designed to minimize the number of animals used while still providing scientifically valid results? Provide adequate statistical justification, ie. power calculations, or other information to justify the number of requested animals including a detailed explanation of the experimental design. The contract calls for 50 sheep at all times. Since scrapie is a slow and complex disease and the experiment depends on natural infection, large numbers of animals are needed to assure an adequate supply of infected animals throughout the year. It is estimated that fifty infected sheep are required over a 2 year period and only about 10% are clinical at any one time. Further, we need to replace those that are euthanized for terminal scrapie. Since only 5-10 animals may be clinical over the year, we need 50 animals per year to maintain the number of clinical sheep needed.

4. STUDY AREAS

Will animals be taken to a laboratory or other area and maintained for longer than 12 hours? ☐ Yes ☒ No

If yes, list the room number and building.

5. NAME OF VETERINARIAN (if other than the attending veterinarian for the facility)

6. ANIMAL USE PROCEDURES (Respond to all items. For all yes responses, provide descriptions and additional information in the blanks provided. Attach additional sheet, if required.)

YES NO

☒ ☐

Blood sampling (describe techniques, site of collection, volumes, frequency)

In live animal, 20 cc taken from the jugular into 2 10 cc vacutainers tubes using a 18 gauge needle monthly in some, and annually in others. The area of the jugular is palpated, and needle is inserted in the jugular. After the sample is collected manual pressure is placed over the needle insertion site to prevent bleeding.

☒ ☐

Sampling of urine/feces (indicate method, e.g., metabolism cage, including dimensions of cage, catheterization, frequency of catheterization, other) Urine will be collected occasionally, never more than monthly. Metabolism cages are 60" long, 22" wide and 24" tall, the sheep



will be kept in them no longer than 3 h at a time, without catheterization. Water will be available while the sheep are in the crates.

- ☐ ☒ Antibody production (indicate route of administration, volume administered per site, number of sites, adjuvant use and frequency, collection protocol, consideration of alternatives to Freund's adjuvant)
- ☐ ☒ Administration of drugs (other than anesthetics or analgesics)/reagents/cells/etc. (describe agent, route of administration and frequency, anticipated side effects, monitoring protocol)
- ☒ ☐ Administration of anesthetics (agent, dose, route; if by inhalation, state method of scavenging waste anesthetic gases/fumes)  
Xylazine (0.1-0.4 mg/lb) IV, ketamine (3-6 cc) IM, in combination. Ketamine is kept in controlled drug safe behind two locked doors.  
Third eyelid biopsy- one to two drops 0.5% proparacaine hydrochlorideophthalmic solution will be placed in the eye to be sampled 5-6 min prior to the biopsy.
- ☒ ☐ Administration of analgesics (agent, dose, route, frequency). 1.5-6ml of flunixin meglumine will be administered SQ half hour prior to surgical procedure and another dose 7 h later.
- ☒ ☐ Infectious/potentially infectious agents to humans and/or animals used (describe arrangements for use)  
These animals are kept separated from all other animals. Personnel change clothes and wash footwear before entering non scrapie areas.
- ☒ ☐ Controlled substance, including anesthetics, e.g., pentobarbital (describe arrangements for use/security).  
Ketamine and pentobarbital kept under double lock in a container attached to the building, behind two locked doors.
- ☒ ☐ Collection of tissues post euthanasia  
Brain, retropharyngeal, mandibular, mesenteric lymph nodes, tonsil, rectal mucosa, kidney, vagus nerve, eyeballs, spleen, ileum and lymphoid tissue of the third eyelid. Personnel will wear aprons, boots, goggles and gloves during necropsy and tissue removal.
- ☐ ☒ Special diets (describe any anticipated nutritional deficit)
- ☐ ☒ Indwelling catheters or implants (describe size, type, maintenance/monitoring protocol)
- ☒ ☐ Restraint (describe method, duration) Chemical restraint for surgery (see above) and manual restraint by a technician holding the head for blood sampling.



- \_\_\_ X Behavioral testing *without* significant restraint or noxious stimuli
- \_\_\_ X Behavioral testing *with* significant restraint or noxious stimuli (describe; provide rationale for degree or restraint of stimulus)
- \_\_\_ X Tumor transplantation (describe any anticipated functional deficit to the animal, monitoring protocol, endpoint)
- \_\_\_ X Toxicity testing and toxic materials (describe procedure, anticipated side effects, endpoint)
- \_\_\_ X Nonsurvival surgery (if yes, complete Animal Surgery page)
- X \_\_\_ Single survival surgery (If yes, complete Animal Surgery page) Rectal cutaneous mucosal biopsy snips performed every six months in order to determine when scrapie can be detected in the preclinical animal. This may be done twice during the lifetime of the animal.
- \_\_\_ X Multiple major survival surgeries involving an individual animal. Multiple major survival surgery is defined as one surgery which penetrates and exposes a body cavity, followed by a second survival surgery which also penetrates and exposes a body cavity or debilitates the animal. (If yes, complete Animal Surgery page)
- \_\_\_ X Procedures involving potential pain, where pain-relieving methods will not be used (completely describe procedures; explain why alternatives are scientifically inappropriate in your written justification for a Type E procedure)
- \_\_\_ X Radioisotopes (describe arrangements for use)

7. **METHOD OF EUTHANASIA** (specify method, agent, dosage, and route of administration that will be used either during the normal course of the protocol or in emergencies; the euthanasia method used must be consistent with the AVMA Panel on Euthanasia, Journal of Veterinary Medical Association (Volume 218, No. 5, March 1, 2001), or justification for deviation should be indicated).

Anesthesia by .2 mg/lb xylazine plus 5 cc ketamine, followed by exsanguination.

8. If animals are not euthanized, indicate their disposition. NA



9. **ALTERNATIVE TO LIVE ANIMAL USE**

Federal law requires that the principal investigator **CONSIDER ALTERNATIVES TO PROCEDURES THAT MAY CAUSE MORE THAN MOMENTARY OR SLIGHT PAIN OR DISTRESS TO ANIMALS** covered by the Animal Welfare Act and Public Health Service Policy. In order to assure compliance with these regulations, the ACUC requires the following information for all protocols with type D or E animal use.

Do in vitro, non-invasive or non-surgical alternatives to the proposed procedures exist? If yes, state why these alternatives are unacceptable. no

If the project involves survival surgery, do models not requiring survival surgery exist? If yes, justify the use of survival surgery. no

A written narrative of the results of your search efforts as they pertain to the study is required. Provide information on the methods used and sources consulted to determine the answers to the above questions and how the search results pertain to your study. As a minimum, the database(s) used to search the literature, the date the search was performed, the date ranges searched, the keywords used, and the results obtained should be listed. Additional clarification on what qualifies as valid information for an alternatives search when a database is not used may be found in USDA's Policy 12,

<http://www.aphis.usda.gov/ac/policy/policy12.pdf>.

Searched Internet Medlin, Promed-scrapie, CJD, BSE, TSE. Done 4/16/06, from 1994 (as early as the search engines go)

There have been no models yet devised to study prion disease outside a living animal. No plant or micro-organism has yet been found to harbor scrapie prions.

The rectal and third eyelid biopsy procedures are the least invasive of the possibilities for getting a lymphoid sample from a live animal. The sheep show no sign of discomfort or distress after the procedure: no rubbing, crying, straining and they return to eating immediately. The article (attached) by Espenes states that "minimal discomfort as judged by the absence of signs of distress" was noted following rectal mucosal biopsy and no analgesics were used. Anesthesia for third eyelid biopsy will be as described above.



10. **PERSONNEL QUALIFICATIONS**

Personnel actively involved with animal components of the project and qualification (as a minimum, should include principal investigator, research technicians, TA, graduate students, etc.)

Individual	Component of project with which involved (e.g., injection, collection of samples, etc.)	Qualifications/ Training/AALAS Certification
Marie Bulgin	Sample collection	Veterinarian
Sharon Melson	Sample collection, routine care, record keeping	MS, research associate
Trisha Walker	Sample collection, routine care, feeding	Vet technician assistant
Brian Meyer	Sample collection, routine care, feeding	Technician

11. Have personnel involved with project attended the seminars on animal use presented by the Institutional Animal Care and Use Committee:

☒ Yes      ☐ No If yes, date 1996, 2006

**PRINCIPAL INVESTIGATOR ASSURANCE**

The information contained on this form provides an accurate description of the animal care and use protocol which will be followed. I agree to abide by governmental regulations and university policies concerning the use of animals. I will allow veterinary care to be provided to animals showing evidence of pain or illness. If the information provided for this project concerning animal use should be revised, or procedures changed, I will so notify the committee of those changes. All proposed changes will not be implemented until full IACUC approval has been granted. I understand that failure to report significant changes may place the university and myself in violation of federal regulations.

As required by federal regulations, *the activities described do not unnecessarily duplicate previous experiments.*

*Marie S Bulgin*

Signature of Principal Investigator

*4/21/06*

Date

Send original form plus seven copies to the University Research Office, 111 Morrill Hall (885-6651).



## ANIMAL SURGERY INFORMATION

Name of All Participating Surgeons, Technicians and Students	Indicate Certification by Either Training or Experience--Explain
Marie Bulgin	Veterinarian
Jason Down	Veterinarian
Sharon Melson	Research associate
Trisha Walker	Vet technician asst
Brian Meyer	Animal technician
Susan Lindstedt	Lab assistant

1.

Species Used	Number Used	S = Survival N = Nonsurvival*	Building/Room Where Surgery Performed
Ovine	50	S	Caine Veterinary Teaching Center

\*Non-survival surgery animal not allowed to awaken, once anesthetized

2. Check following procedures that apply. If procedure is not listed, please use an additional page and describe completely or attach literature reference article.

☒ Biopsy: target organ/tissue ☐ third eyelid (J of Clinical Microbiology, 2000;38(9):3254-3259), rectal mucosa (J Comparative Pathology 2006; 1-11.) See attached articles.

Laparotomy ☐ Intracranial ☐ Thoracotomy ☐ Orthopedic

☐ Other \_\_\_\_\_

3. PREOPERATIVE PROCEDURES

Have unhealthy animals been exempted for surgery? ☐ Yes ☒ No

Person responsible for evaluating health status of animals: Dr. Marie Bulgin

4. ANESTHETIC PROCEDURES

Drug	Dose	Route	Duration
Xylazine	.1-4 mg/lb	IV	30-45 min
Ketamine	3-6 cc	IM	30-45 min
0.5 % Proparacaine hydrochloride ophthalmic	1-2 drops	Into eye	30-45 min







UNIVERSITY OF MARYLAND  
SCHOOL OF MEDICINE  
Animal Care and Use Office

685 W. Baltimore Street  
HSF 1, Room 146  
Baltimore, MD 21201-1559

email: ACUO@som.umaryland.edu  
voice: (410) 706-4365  
Assurance Number: A3200-01

DATE: June 25, 2004

TO: Robert Rohwer, Ph.D.  
Department of Neurology  
BVAMC, c/o Research Office, 3rd Floor

FROM: Institutional Animal Care and Use Committee

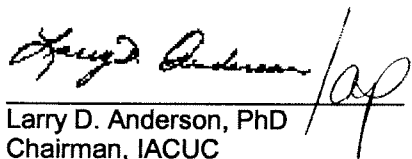
RE: IACUC PROTOCOL #0304010  
"Use of Transgenic Mice to Titrate Naturally Occuring TSE Infectivity from  
Other Species"

---

This is to certify that the Institutional Animal Care and Use Committee received your response to their queries and that your response was considered sufficient to grant FULL APPROVAL to your protocol. Your approval date for this protocol is March 19, 2004.

Your protocol is approved for a period of 3 years; an annual report must be submitted to the IACUC one month before each anniversary of the protocol. Please note that your protocol will expire on March 19, 2007. If you need to extend the protocol beyond this date, you must submit an ARF at least 3 months prior to the expiration.

*If you have any questions, please do not hesitate to contact the Animal Care and Use Office by email (ACUO@som.umaryland.edu) or by phone (at 706-4365).*

  
Larry D. Anderson, PhD  
Chairman, IACUC



**Research Service, VA Maryland Health Care System  
10 N. Greene Street, Baltimore, MD 21201**

**MEMORANDUM**

DATE: May 7, 2004

TO: Institutional Animal Care and Use Committee

FROM: Robert Rohwer, Ph.D.  
Department of Neurology, UMMS & Research Service, VAMHCS

RE: IACUC PROTOCOL # 0304010

“Use of Transgenic Mice to Titrate Naturally Occurring TSE Infectivity from Other Species”

---

This memo is in response to the questions of the committee about the above protocol.

1. *“There is a discrepancy in the number of animals requested per strain in the ARF (990) versus the narrative (330). Please clarify and revise accordingly.”*

The total number of animals per strain should be 990. The confusion is a result of how we presented the numbers in the tables provided. We will attempt to clarify the tables that were provided in Attachment C-Justification for the Number of Animals. For each strain we describe that we will be performing titrations (both End-Point and Limiting) with a different isolate sample per year. The reasoning for this was given in the narrative as follows:

“We will test one brain sample per year from various isolates from each strain of TSE in each strain of transgenic mouse. There is evidence of wide variation in environmental isolates of each strain of TSE (there are unknown numbers of isolates in CWD) which is why we are choosing to experiment with various isolates from each strain of TSE in each strain of transgenic mouse..... We will test one brain sample per year and one blood sample per year from various isolates of TSE for each transgenic mouse strain.”

A clarified tabular summary is corrected in the narrative and follows:



1. **Sheep Scrapie Brain titration in sheep transgenic mice**

End-Point Dilution Titration - one sample per year

Inoculum	# of Inoculated Animals	
	Titration 1	Titration 2
$10^{-1}$	5	5
$10^{-2}$	5	5
$10^{-3}$	5	5
$10^{-4}$	5	5
$10^{-5}$	5	5
$10^{-6}$	5	5
$10^{-7}$	5	5
$10^{-8}$	5	5
$10^{-9}$	5	5
$10^{-10}$	5	5
$10^{-11}$	5	5
Uninoculated control	5	5
Per-Year Sub-Total	60	60
Per-Year Total		120
<i>One sample per year</i>		<i>3 years</i>
<b><i>Study Grand Total</i></b>		<b><i>360</i></b>

2. **Sheep Scrapie Blood titration in sheep transgenic mice**

Limiting Dilution Titration - one sample per year

Inoculum	# Inoculated Animals
Blood from scrapie-infected sheep	200
Uninoculated controls	10
Per-Year Total	210
<i>One sample per year</i>	<i>3 years</i>
<b><i>Study Grand Total</i></b>	<b><i>630</i></b>



3. **Human CJD and vCJD Brain Titration in human transgenic mice**  
End-Point Dilution Titration – one sample per year

Inoculum	# Inoculated Animals	
	Titration 1	Titration 2
$10^{-1}$	5	5
$10^{-2}$	5	5
$10^{-3}$	5	5
$10^{-4}$	5	5
$10^{-5}$	5	5
$10^{-6}$	5	5
$10^{-7}$	5	5
$10^{-8}$	5	5
$10^{-9}$	5	5
$10^{-10}$	5	5
$10^{-11}$	5	5
Un-inoculated control	5	5
Per-Year Sub-Total	60	60
Per-Year Total		120
<i>One sample per year</i>		<i>3 years</i>
<b><i>Study Grand Total</i></b>		<b><i>360</i></b>

4. **Human CJD and vCJD Blood Titration in human transgenic mice**  
Limiting Dilution Titration – one sample per year

Inoculum	# Inoculated Animals
Human CJD blood	200
Uninoculated controls	10
Per-Year Total	210
<i>One sample per year</i>	<i>3 years</i>
<b><i>Study Grand Total</i></b>	<b><i>630</i></b>



**5. Mule Deer/white tail deer/elk CWD Brain Titration in elk transgenic mice**  
End-Point Dilution Titration- one sample per year

Inoculum	# Inoculated Animals	
	Titration 1	Titration 2
10 <sup>-1</sup>	5	5
10 <sup>-2</sup>	5	5
10 <sup>-3</sup>	5	5
10 <sup>-4</sup>	5	5
10 <sup>-5</sup>	5	5
10 <sup>-6</sup>	5	5
10 <sup>-7</sup>	5	5
10 <sup>-8</sup>	5	5
10 <sup>-9</sup>	5	5
10 <sup>-10</sup>	5	5
10 <sup>-11</sup>	5	5
Un-inoculated control	5	5
Per-Year Sub-Total	60	60
Per-Year Total		120
<i>One sample per year</i>		<i>3 years</i>
<b><i>Study Grand Total</i></b>		<b><i>360</i></b>

**6. Mule Deer/white tail deer/elk CWD Blood Titration in elk transgenic mice**  
End-Point Dilution Titration – one sample per year

Inoculum	# Inoculated Animals
CWD blood	200
Un-inoculated controls	10
Per-Year Total	210
<i>One sample per year</i>	<i>3 years</i>
<b><i>Study Grand Total</i></b>	<b><i>630</i></b>

2. “Regarding the IC inoculations of blood, please indicate the dose of Pentobarbital to be used for re-dosing if needed. Standard dose for re-dose is 50% of original dose used.”

If re-dosing is needed, our standard protocol is 50% of the original dose, in this case 20-30 mg/kg body weight.

3. “Please clarify how long animals will be monitored after IC inoculation.”



If there is any immediate affect from the inoculation animals will be monitored constantly for the first hour, then on an hourly basis throughout the day.

4. *"Under biohazardous materials, it is stated that all waste will be autoclaved for 1-2 hours at 134 degrees Celsius, and an SOP is referenced, but not provided. Please provide a copy of the SOP or remove the reference from the protocol."*

We will remove the reference from the protocol.

5. *"There is a discrepancy between the ARF and narrative relative to the animal biosafety level to be used. Please clarify and revise accordingly."*

We believe there is no discrepancy between the ARF and narrative regarding the biosafety level of the agents used. The confusion may be that we operate our animal facility under biosafety level 3 (as we detail in the narrative) conditions at all times, but the agents being used are either biosafety level 2, or unclassified.

The ARF (Section V. Biohazard information, Line A of each strain) identifies Scrapie, CJD, and <sup>var</sup>CJD as biosafety level 2 agents. The ARF identifies CWD as unclassified. This is further detailed in item L. Biohazardous Materials of the narrative as follows:

"Scrapie, CJD, and <sup>var</sup>CJD are classified as Biosafety Level II agents. Chronic Wasting Disease is unclassified. Scrapie and chronic wasting disease have unknown potential for human disease. Although it is not required to work with these agents in a BL3 laboratory, the principal investigator prefers to work with these agent in a restricted environment, until the potential, or lack thereof, for human disease are more firmly established. Therefore, all mice used for studies with material infected with the above three strains will be maintained in the VA Animal Biosafety Level Three Laboratory, ABSL3."

6. *"Please conduct an updated search for Agricola/Biosis."*

While the Agricola/Biosis search is very thorough, it is very expensive. The USDA library in Beltsville, MD provides us the ability to search at no charge once a year. We will continue to update our Agricola/Biosis searches yearly. For this protocol, we have added two additional search engines that are aimed at finding duplications and animal topics. These are:

- a) Biomedical Research Database (BRD) at  
<http://www.scitechweb.com/acau/brd/>
- b) Computer Retrieval of Information of Scientific Projects (CRISP) at  
<http://www.crisp.cit.nih.gov/>



Searches with the same parameters as stated in the ARF were performed with both databases in May 2004.

7. *"This study has been identified as having to be reviewed by VA Biosafety Committee due to use of infectious agents. Please note that IACUC approval cannot be granted until VA Biosafety Committee approval has been received. Please forward one copy of their approval letter for the IACUC file."*

Please find attached a copy of the VA Biosafety Committee approval

8. *"As a result of NIH's policy on "just in time" IACUC approval, verification of protocol and grant congruency may not occur at the time of routing as an approved protocol may not exist. As such, this task must be completed by the ACUO. Please provide one copy of the FUNDED grant for these purposes. The grant will be filed with the protocol. All information is treated as confidential. All unnecessary information will be shredded. If the grant is still undergoing NIH review or has not been submitted yet, please indicate such and provide a final copy for the IACUC file if funded."*

Please find attached a copy of the NIH contract.



INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE  
ANIMAL RESEARCH FORM (ARF)  
--INFORMATION MUST BE TYPED--

Principal Investigator: Robert G. Rohwer, Ph.D. Degree: Ph.D. SS#: 478-54-0738  
Address: VA Medical Center, Baltimore, 10 N. Greene St. Dept: Neurology Phone: 410-605-7000, x6462  
E-mail: rrohwer@umaryland.edu Emergency Contact: Rene'e Kahn Phone: 410-605-7000, x6487  
Project Title: Use of transgenic mice to titrate naturally-occurring TSE infectivity from other species

Co-Investigators:			Technicians:		
Name	Dept	Phone	Name	Dept	Phone
<u>Rene'e Kahn, B.A.</u>	<u>VAMC/Research</u>	<u>X6487</u>	<u>Kris Earll, Willie Paige, ALAT</u>	<u>VAMC/Research</u>	<u>6418</u>
<u>Claudia MacAuley, ALAT</u>	<u>VAMC/Research</u>	<u>X6544</u>	<u>Annette Wise</u>	<u>"</u>	<u>6418</u>
_____	_____	_____	_____	_____	_____
_____	_____	_____	_____	_____	_____

I. RESEARCH CATEGORIES

Major Categories of Research Use: Please check as applicable. All "checked" responses must be completely addressed in the NARRATIVE attachment.

- A. ☒ Euthanize and Harvest Tissue (detail method of euthanasia)  
B. ☐ Immunization/Antibody Production (include antigen, adjuvant use, route of immunization, method of obtaining blood as well as volume & frequency)  
C. ☐ Physiologic Measurements (provide detailed descriptions)  
D. ☐ Dietary Manipulations (food or water restriction, special diets, provide details on parameters, monitoring and justify, etc.)  
E. ☐ Pharmacology/Toxicology (materials used, dose, route of administration, frequency, duration, endpoint, etc.)  
F. ☐ Investigational Drug (provide company drug information sheets and/or list of possible side effects to animals, etc.)  
G. ☐ Behavioral Studies (provide detailed description)  
H. ☐ Trauma (provide a detailed description)  
I. ☐ Oncology/Tumor Transplantation (provide information on origin, passage, adventitious pathogen testing [MAP], biohazard potential, endpoint, etc.)  
J. ☐ Sampling (tissue/substance, amount, frequency, method, etc.)  
K. ☒ Dosing (agent, dose, route of administration, frequency, duration, etc.)  
L. ☐ Breeding Colony (justify need)  
M. ☒ Biohazardous /Infectious Agents (describe the nature of hazard and personnel safety precautions)  
N. ☐ Chronic or Prolonged Restraint (provide justification for restraint, a description of the device and duration of the restraint)  
O. ☐ Surgery  
    ☐ Survival Surgery  
    ☐ Non-Survival Surgery  
    ☐ Multiple Major Survival Surgery: species \_\_\_\_\_ (Same animal surviving two or more surgeries)  
    Provide adequate justification for need.  
P. ☒ Specialized Housing/Husbandry (contact Veterinary Resources, x 63540 and describe arrangements)  
Q. ☐ Teaching

II. CATEGORY OF PAIN

DEFINITIONS of each category are given on page 1 of the instruction sheets.

A. ☐ B. ☒ C. ☐

III. FUNDING SOURCE

CURRENT OR ANTICIPATED

- ☒ PHS  
☐ NSF  
☐ State of Maryland  
☐ Departmental/Internal Funds  
☐ Other External Funds (specify) \_\_\_\_\_

ARF 5/01



#### IV. ANIMAL USAGE

Species/Strain	Weight/Age	Sex	(# PER YEAR)			Max Daily Census
			YR 1	YR 2	YR3	
FVB mouse, transgenic for sheep PrP gene	weanling	m & f	330	330	330	400

III ADDITIONAL SPECIES/STRAINS ARE BEING REQUESTED, INCLUDE ON AN ADDITIONAL PAGE

#### V. BIOHAZARD INFORMATION

Please indicate the general biohazard being used in vivo:

Infectious Agents X      Radioactive Substances \_\_\_\_\_      Toxic Chemicals/ Chemical Carcinogens \_\_\_\_\_      Recombinant DNA \_\_\_\_\_

Others (specify) \_\_\_\_\_

Name of Agent scrapie (natural scrapie from sheep)  
 Please provide: A) ABL level (1, 2, or 3) of infectious agents ABL2  
 B) Concentration 10<sup>9</sup> to 10<sup>10</sup> infectious units/ml (estimated)  
 C) Route of administration intra-cranial inoculation  
 D) Duration of exposure during inoculation, less than 20 seconds; during incubation, 3 to 24 months  
 E) Room location where agent is administered BB-113 (within the BL3 facility)  
 F) Location of animal housing post exposure BB-113 (within the BL3 facility)  
 G) Length of time animals will be kept following exposure duration of animal's life; 3 to 24 months  
 H) Method of animal disposal incineration at biohazardous waste disposal facility

#### VI. SURGERY

##### Survival Surgery \*

A. Procedure \_\_\_\_\_ Species \_\_\_\_\_  
 B. Anesthesia \_\_\_\_\_  
 C. Building & Room Number where surgery will take place \_\_\_\_\_  
 D. Person performing survival surgery \_\_\_\_\_  
 E. Post-Operative Care (e.g., supportive fluids, analgesics, antibiotics, other drugs & frequency of observation) \_\_\_\_\_  
 F. Person(s) providing & recording post-operative care \_\_\_\_\_

\*Describe in detail the surgery, aseptic procedures & post-operative care in the NARRATIVE section.

##### Non-Survival Surgery

A. Procedure \_\_\_\_\_ Species \_\_\_\_\_  
 B. Anesthesia \_\_\_\_\_  
 C. Method of Euthanasia \_\_\_\_\_  
 D. Building & Room Number where surgery will take place \_\_\_\_\_  
 E. Person(s) performing non-survival surgery \_\_\_\_\_

#### VII. METHOD OF EUTHANASIA

SPECIES	AGENT	DOSE (mg/kg)	ROUTE	METHOD
<u>Mouse</u>	<u>gaseous CO<sub>2</sub></u>	<u>30 sec to unconsciousness;</u> <u>2 min to death</u>	<u>inhalation</u>	<u>Animal is placed in chamber into which gaseous CO<sub>2</sub> is introduced, until animal loses consciousness and ceases breathing. Death is confirmed by immediate removal of brain.</u>

#### IV. ANIMAL USAGE



Species/Strain	Weight/Age	Sex	(# PER YEAR)			Max Daily Census
			YR 1	YR 2	YR3	
FVB mouse, transgenic for elk Prp gene	weanling	m & f	330	330	330	400

IF ADDITIONAL SPECIES/STRAINS ARE BEING REQUESTED, INCLUDE ON AN ADDITIONAL PAGE

#### V. BIOHAZARD INFORMATION

Please indicate the general biohazard being used in vivo:

Infectious Agents <u>X</u>	Radioactive Substances _____	Toxic Chemicals/ Chemical Carcinogens _____	Recombinant DNA _____
----------------------------	------------------------------	---	-----------------------

Others (specify) \_\_\_\_\_

Name of Agent Chronic Wasting Disease (CWD)

Please provide:

- A) ABL level (1, 2, or 3) of infectious agents Unclassified
- B) Concentration 10<sup>9</sup> to 10<sup>10</sup> infectious units/ml
- C) Route of administration intra-cranial inoculation
- D) Duration of exposure during inoculation, less than 20 seconds; during incubation, 3 to 24 months
- E) Room location where agent is administered BB-113 (within the BL3 facility)
- F) Location of animal housing post exposure BB-113 (within the BL3 facility)
- G) Length of time animals will be kept following exposure duration of animal's life; 3 to 24 months
- H) Method of animal disposal incineration at biohazardous waste disposal facility

#### VI. SURGERY

##### Survival Surgery \*

- A. Procedure \_\_\_\_\_ Species \_\_\_\_\_
- B. Anesthesia \_\_\_\_\_
- C. Building & Room Number where surgery will take place \_\_\_\_\_
- D. Person performing survival surgery \_\_\_\_\_
- E. Post-Operative Care (e.g., supportive fluids, analgesics, antibiotics, other drugs & frequency of observation) \_\_\_\_\_
- F. Person(s) providing & recording post-operative care \_\_\_\_\_

\*Describe in detail the surgery, aseptic procedures & post-operative care in the NARRATIVE section.

##### Non-Survival Surgery

- A. Procedure \_\_\_\_\_ Species \_\_\_\_\_
- B. Anesthesia \_\_\_\_\_
- C. Method of Euthanasia \_\_\_\_\_
- D. Building & Room Number where surgery will take place \_\_\_\_\_
- E. Person(s) performing non-survival surgery \_\_\_\_\_

#### VII. METHOD OF EUTHANASIA

SPECIES	AGENT	DOSE (mg/kg)	ROUTE	METHOD
<u>Mouse</u>	<u>gaseous CO<sub>2</sub></u>	<u>30 sec to unconsciousness;</u> <u>2 min to death</u>	<u>inhalation</u>	<u>Animal is placed in chamber into which gaseous CO<sub>2</sub> is introduced until animal loses consciousness and ceases breathing. Death is confirmed by immediate removal of brain.</u>

#### IV. ANIMAL USAGE



Species/Strain	Weight/Age	Sex	# PER YEAR			Max Daily Census
			YR 1	YR 2	YR3	
FVB mouse, transgenic for human Prp gene	weanling	m & f	330	330	330	400

IF ADDITIONAL SPECIES/STRAINS ARE BEING REQUESTED, INCLUDE ON AN ADDITIONAL PAGE

## V. BIOHAZARD INFORMATION

Please indicate the general biohazard being used in vivo:

Infectious Agents X      Radioactive Substances \_\_\_\_\_      Toxic Chemicals/ Chemical Carcinogens \_\_\_\_\_      Recombinant DNA \_\_\_\_\_

Others (specify) \_\_\_\_\_

Name of Agent Creutzfeldt-Jakob Disease (CJD) and variant Creutzfeldt-Jakob Disease (var CJD)

Please provide: A) ABL level (1, 2, or 3) of infectious agents ABL2  
 B) Concentration 10<sup>9</sup> to 10<sup>10</sup> infectious units/ml  
 C) Route of administration intra-cranial inoculation  
 D) Duration of exposure during inoculation, less than 20 seconds; during incubation, 3 to 24 months  
 E) Room location where agent is administered BB-113 (within the BL3 facility)  
 F) Location of animal housing post exposure BB-113 (within the BL3 facility)  
 G) Length of time animals will be kept following exposure duration of animal's life; 3 to 24 months  
 H) Method of animal disposal incineration at biohazardous waste disposal facility

## VI. SURGERY

### Survival Surgery \*

A. Procedure \_\_\_\_\_ Species \_\_\_\_\_  
 B. Anesthesia \_\_\_\_\_  
 C. Building & Room Number where surgery will take place \_\_\_\_\_  
 D. Person performing survival surgery \_\_\_\_\_  
 E. Post-Operative Care (e.g., supportive fluids, analgesics, antibiotics, other drugs & frequency of observation) \_\_\_\_\_  
 F. Person(s) providing & recording post-operative care \_\_\_\_\_

\*Describe in detail the surgery, aseptic procedures & post-operative care in the NARRATIVE section.

### Non-Survival Surgery

A. Procedure \_\_\_\_\_ Species \_\_\_\_\_  
 B. Anesthesia \_\_\_\_\_  
 C. Method of Euthanasia \_\_\_\_\_  
 D. Building & Room Number where surgery will take place \_\_\_\_\_  
 E. Person(s) performing non-survival surgery \_\_\_\_\_

## VII. METHOD OF EUTHANASIA

SPECIES	AGENT	DOSE (mg/kg)	ROUTE	METHOD
<u>Mouse</u>	<u>gaseous CO<sub>2</sub></u>	<u>30 sec to unconsciousness;</u> <u>2 min to death</u>	<u>inhalation</u>	<u>Animal is placed in chamber into which gaseous CO<sub>2</sub> is introduced, until animal loses consciousness and ceases breathing. Death is confirmed by immediate removal of brain.</u>

## VIII. LOCATIONS OF ANIMAL USAGE



Please list all locations where Animal Procedures will be performed & check the appropriate blank. These areas will be inspected, randomly, on a semiannual basis. It is preferred, when possible, that procedures be performed in the Animal Facility Procedure Rooms.

ANIMAL FACILITIES

Building	Floor/Room #	LABORATORY	PROCEDURE ROOM	TYPE OF PROCEDURE
1	MC, BL3 facility, Rm BB-113	_____	<u>  X  </u>	<u>Housing of mice, incubation of disease</u>
2.	Rm BB-113	_____	<u>  X  </u>	<u>inoculation, euthanasia of mice</u>

\*\*Animals may not be housed in an investigator's laboratory for more than 24 hours unless specifically approved by the IACUC. Submit documentation for this approval with this application.\*\*



## IX. DATABASE LITERATURE SEARCH

Identify the services (computer databases, literature searches, etc.) that were used to obtain information on alternatives to painful procedures, use of live animals and prevention of unnecessary duplication of research.

Please check below the databases searched and your search strategy or key words. A MINIMUM OF TWO SEPARATE DATABASES MUST BE USED. Please do not submit the actual search results (however, they should be available upon request). (Refer to instructions for examples.)

DATE OF SEARCH: February 2004 for Medline; November 2003 for Agricola/Biosis; May 2004 Biomedical Research Database (BRD); May 2004 Computer Retrieval of Information of Scientific Projects (CRISP)

DATABASES: MEDLINE X; AGRICOLA X; EMBASE \_\_\_\_\_; PSYCHINFO \_\_\_\_\_; OTHER BIOSIS, BRD & CRISP

- 1) STRATEGY OR KEY WORDS: scrapie OR Creutzfeld-Jakob Disease OR bovine spongiform encephalopathy OR transmissible spongiform encephalopathy OR chronic wasting disease OR prion disease OR prions OR PrP; Alternatives to Animals AND scrapie OR prion disease OR transmissible spongiform encephalopathy; Tissue Culture AND scrapie OR prion disease OR transmissible spongiform encephalopathy; Transgenic animals AND scrapie OR Creutzfeld-Jakob Disease OR bovine spongiform encephalopathy OR transmissible spongiform encephalopathy OR chronic wasting disease OR prion disease OR prions OR PrP

DATE PARAMETERS OF SEARCH: 1966 to February 2004

### AREAS OF RESPONSIBILITY

Veterinary Resources is responsible for maintaining programs of laboratory animal care, including animal procurement, husbandry, disease control and prevention, humane treatment and adequate veterinary care under the supervision of a doctor of veterinary medicine.

The Principal Investigator is responsible for all aspects of the research protocol including post-operative monitoring and care, research related complications, and humane treatment by investigative personnel.

### PRINCIPAL INVESTIGATOR'S ACKNOWLEDGMENT OF RESPONSIBILITY

I certify that the activities described in this protocol do not unnecessarily duplicate previous experiments.

I certify the above protocol will be conducted in compliance with the Federal, State, and local policies and regulations. I also acknowledge full responsibility for knowledge of and compliance with all applicable standards governing radioactive or biohazardous materials involved in my project. I understand that compliance with these policies is a prerequisite for purchasing and housing animals, and for the use of animals in research and teaching at UMB School of Medicine.

\_\_\_\_\_  
Signature

Robert G. Rohwer, Ph.D.

\_\_\_\_\_  
Printed Name

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature - VA Research Office (if animal work will be done in the VA)

**\*NOTE\*** The IACUC requires one (1) original and fifteen (15) copies in order to process the proposal for full committee review.



## **Attachment A - Summary of the study**

We propose here to use three new transgenic strains of mice, in which the gene for the prion protein, or PrP, from humans, sheep, and elk has been inserted into the mice, replacing the mouse PrP gene. The prion gene controls an individual's susceptibility to transmissible spongiform encephalopathy (TSE) disease and creates a barrier to transmission between species. This barrier can sometimes be overcome by changes in the infecting agent over several passages in the recipient strain, or it can be overcome with the use of transgenic animals. A PrP transgenic mouse is more likely to be infectable by the TSE disease that naturally infects the donor of the transgene. If successful, this work will aid the development of TSE diagnostics and TSE research in general by providing a means of directly measuring the infectivity in TSE strains that normally only infect large animals or humans.

Because bioassay is currently the only sensitive assay for transmissible spongiform encephalopathy (TSE) disease, and because bioassay is impossible in humans and impractical in sheep, and elk (the commercially important species affected by TSE diseases), it is critical to develop strains of laboratory animals, which can be used to assay these TSE diseases. This is most readily done using transgenic technology. So far, only a few of the many transgenic mouse strains developed have sufficient sensitivity to TSE diseases to be useful for bioassay, and none of these mice have been made generally available. An immediate objective is to be able to measure the titer of TSE infectivity in blood and other tissues of public health and commercial significance in these species. We will use these animals for bioassay of blood and brain samples from sheep infected with scrapie, deer infected with CWD (chronic wasting disease of deer and elk), and humans infected with CJD (Creutzfeldt-Jakob Disease) & vCJD (variant Creutzfeldt-Jakob Disease).

The transgenic mice have been developed by Dr. Richard Rubenstein, a collaborator at the Institute for Basic Research in Staten Island, NY. We have made arrangements with Dr. Rubenstein and the IBR to have the company Harlan Sprague Dahley breed these three transgenic strains and provide our laboratory with them as needed.

## **Attachment B – Justification for the use of animal and species**

### **Rational for using animals**

Currently there is no substitute for an animal bioassay for the sensitive detection of TSE infectivity. Progress has been made in growing TSE diseases in tissue culture, but the cultures are not suitable for assay purposes because they are unpredictable and difficult to infect. There are several biochemical assays for the infection-specific form of the PrP protein- a Western blot, and several ELISA-based assays-, which are used for biochemical confirmation of disease in bioassay animals that have progressed to full clinical symptoms. However, these assays are several orders of magnitude less sensitive than the bioassay, and cannot detect low levels of infectivity present in most tissues or during preclinical infection. Moreover, it is not known for sure whether these assays are measuring infectivity or only a surrogate marker and whether the



latter tracks with infectivity in all cases. This experiment will test if the transgenic mice supplied by Dr. Rubenstien are susceptible to scrapie, CWD, CJD, and vCJD. If successful, these animals may eventually be used as a bioassay for TSE in humans, sheep, and elk.

### **Appropriateness of the species selected**

The natural hosts of TSE disease are large animals with very long incubation times, making it impractical and prohibitively expensive to use the natural host to study the disease. The use of mice is necessary in order to study strains of TSE disease that may be more clinically relevant than scrapie, which is the most commonly used TSE model disease.

The use of transgenic mice, in which specific genes from other species are introduced into the mouse in order to overcome the species barrier to infection with natural TSE diseases, has been useful in the study of TSE diseases. A PrP transgenic mouse is more likely to be infectable by the TSE disease that naturally infects the donor of the transgene. If successful, this work will aid the development of TSE diagnostics and TSE research in general by providing a means of directly measuring the infectivity in TSE strains that normally only infect large animals or humans.

All infected sheep blood, from both naturally occurring infected sheep and inoculated infected sheep, will be acquired from Dr. Marie Bulgin. All infected human blood samples are de-identified and will be acquired from Dr. Antonio Giulivi at the CJD Surveillance Unit of Health Canada. All infected human brain samples are de-identified and will come from Dr. Gambetti at the CJD Surveillance Unit at Case Western University or from our own stocks. Blood infected with CWD will be acquired from Dr. Beth Williams of the Colorado Department of Fish & Wildlife and CWD infected brain will come from Dr. Williams or our own stocks.

## **Attachment C – Justification of number of animals**

### **How the number of animals was determined**

#### *Endpoint dilution titration*

Appropriate brain samples will be tested for infectivity in each transgenic mouse strain (scrapie-infected sheep brain in the sheep transgenic mouse, CJD-infected human brain in the human transgenic mouse, and CWD-infected deer or elk brain in the elk transgenic mouse) with a method called end-point dilution titration. A ten-fold dilution series of brain tissue homogenized in phosphate buffered saline (PBS) will be prepared, beginning with a dilution of  $10^{-1}$  and continuing to  $10^{-11}$ . Each of the ten dilutions will be inoculated intracranially (IC) into one cage of mice (5 mice per cage), thus requiring 50 mice per titration. One cage of un-inoculated controls will also be included for each titration. For each titration, a duplicate titration will be performed. In our experience, the variability in titration results is such that a duplicate titration gives a more reliable estimation of the titer. We will test one brain sample per year from various isolates from each strain of TSE in each strain of transgenic mouse. There is evidence of wide variation in environmental isolates of each strain of TSE (there are unknown numbers of



isolates in CWD) which is why we choosing to experiment with various isolates from each strain of TSE in each strain of transgenic mouse.

#### *Limiting dilution titration*

Since the infectivity in blood samples is expected to be many orders of magnitude lower than for brain, blood samples will be assayed by a method our lab has developed, called limiting dilution titration, which differs from end point dilution titration in that the sample being assayed will not be diluted, and in fact is so “dilute” already that not every animal inoculated will become infected. That is, the levels of infectivity in blood from TSE-infected animals are expected to be low enough that not every 25  $\mu$ l aliquot (the maximum volume of inoculum that can be delivered to a mouse) will contain an infectious unit. We have found that, in the case of hamster scrapie, 5 ml of infected blood is the minimum volume that needs to be bioassayed in order to obtain an accurate titer of infectivity in the blood. We propose to assay 5 ml of blood for each of the strains of TSE being tested (sheep scrapie, CWD and CJD). At 25  $\mu$ l of inoculum per mouse, inoculating 5 ml will require 200 mice. There will also be two cages of un-inoculated controls for each limiting dilution titration.

We will test one brain sample per year and one blood sample per year from various isolates of TSE for each transgenic mouse strain. A tabular summary of animal usage is given below.



1. **Sheep Scrapie Brain titration in sheep transgenic mice**

End-Point Dilution Titration - one sample per year

Inoculum	# of Inoculated Animals	
	Titration 1	Titration 2
$10^{-1}$	5	5
$10^{-2}$	5	5
$10^{-3}$	5	5
$10^{-4}$	5	5
$10^{-5}$	5	5
$10^{-6}$	5	5
$10^{-7}$	5	5
$10^{-8}$	5	5
$10^{-9}$	5	5
$10^{-10}$	5	5
$10^{-11}$	5	5
Uninoculated control	5	5
Per-Year Sub-Total	60	60
Per-Year Total		120
<i>One sample per year</i>		<i>3 years</i>
<b><i>Study Grand Total</i></b>		<b><i>360</i></b>

2. **Sheep Scrapie Blood titration in sheep transgenic mice**

Limiting Dilution Titration - one sample per year

Inoculum	# Inoculated Animals
Blood from scrapie-infected sheep	200
Uninoculated controls	10
Per-Year Total	210
<i>One sample per year</i>	<i>3 years</i>
<b><i>Study Grand Total</i></b>	<b><i>630</i></b>



3. **Human CJD and vCJD Brain Titration in human transgenic mice**  
End-Point Dilution Titration – one sample per year

Inoculum	# Inoculated Animals	
	Titration 1	Titration 2
$10^{-1}$	5	5
$10^{-2}$	5	5
$10^{-3}$	5	5
$10^{-4}$	5	5
$10^{-5}$	5	5
$10^{-6}$	5	5
$10^{-7}$	5	5
$10^{-8}$	5	5
$10^{-9}$	5	5
$10^{-10}$	5	5
$10^{-11}$	5	5
Un-inoculated control	5	5
Per-Year Sub-Total	60	60
Per-Year Total		120
<i>One sample per year</i>		<i>3 years</i>
<b><i>Study Grand Total</i></b>		<b><i>360</i></b>

4. **Human CJD and vCJD Blood Titration in human transgenic mice**  
Limiting Dilution Titration – one sample per year

Inoculum	# Inoculated Animals
Human CJD blood	200
Uninoculated controls	10
Per-Year Total	210
<i>One sample per year</i>	<i>3 years</i>
<b><i>Study Grand Total</i></b>	<b><i>630</i></b>



**5. Mule Deer/white tail deer/elk CWD Brain Titration in elk transgenic mice**  
End-Point Dilution Titration- one sample per year

Inoculum	# Inoculated Animals	
	Titration 1	Titration 2
10 <sup>-1</sup>	5	5
10 <sup>-2</sup>	5	5
10 <sup>-3</sup>	5	5
10 <sup>-4</sup>	5	5
10 <sup>-5</sup>	5	5
10 <sup>-6</sup>	5	5
10 <sup>-7</sup>	5	5
10 <sup>-8</sup>	5	5
10 <sup>-9</sup>	5	5
10 <sup>-10</sup>	5	5
10 <sup>-11</sup>	5	5
Un-inoculated control	5	5
Per-Year Sub-Total	60	60
Per-Year Total		120
<i>One sample per year</i>		<i>3 years</i>
<b><i>Study Grand Total</i></b>		<b><i>360</i></b>

**6. Mule Deer/white tail deer/elk CWD Blood Titration in elk transgenic mice**  
End-Point Dilution Titration – one sample per year

Inoculum	# Inoculated Animals
CWD blood	200
Un-inoculated controls	10
Per-Year Total	210
<i>One sample per year</i>	<i>3 years</i>
<b><i>Study Grand Total</i></b>	<b><i>630</i></b>

## Attachment D – Narrative

### Complete narrative description of all animal procedures

#### *Overall Experimental design*

We will obtain weanlings of three strains of transgenic mice with the mouse PrP gene replaced by, respectively, the sheep PrP gene, the elk PrP gene, and the human PrP gene. We will inoculate them intracranially (ic) with brain samples and blood samples from sheep infected with scrapie, deer and elk infected with CWD, and human patients infected with CJD, and vCJD. Inoculated mice will be held until they develop TSE



disease or, if they do not develop disease, for 2 years. Mice will be maintained in the BL3 facility, following our standard practices for housing TSE-inoculated animals.

#### *IC inoculation of brain*

The mice will be anesthetized by ip-injected pentobarbital (40-60 mg/kg body weight) prior to ic inoculation. This step is taken to guard against accidental needle sticks of the inoculator. Mice are minimally affected by the inoculation of brain tissue. Once the animal is unconscious, 25 ul of a 10% homogenate of scrapie, CWD, or CJD brain tissue in phosphate-buffered saline (PBS), will be injected at a point just behind a line between the center of the mouse's ears and a little to the left of the sagittal mid-line. Inoculum will be delivered slowly by a 29 G needle inserted to a depth of 2 to 3mm. This is our standard inoculation procedure. All inoculation procedures will be carried out inside a biosafety cabinet within the BL3 facility.

#### *IC inoculation of blood*

The IC inoculation of blood will be performed in the same way as the IC inoculation of brain (above). Unlike brain IC inoculation, IC inoculation of blood can be noxious to the animal. If there is any immediate affect from the inoculation animals will be monitored constantly for the first hour, then on an hourly basis throughout the day. Animals displaying toxic effects as they recover from anesthesia and will be redosed with pentobarbital (20-30 mg/kg body weight) if necessary (i.e., if they require longer for any side effects of the inoculum to disappear). If animals do not appear normal (normal behavior, body movements, grooming, etc.) after recovering from a second dose of anesthesia, they will be terminated by gaseous CO<sub>2</sub>.

#### *Incubation of Disease*

Animals will be held until they develop TSE disease, or, if they do not develop disease, for at least two years. Animals will receive a daily check to determine that they are alive, healthy, and have sufficient food and water. Once a week, animals will be weighed and inspected more closely for behavioral symptoms of disease onset. Once symptoms are observed in a given mouse, it will be scored daily for TSE symptoms. Animals showing clear signs of TSE (such as weight loss, a scruffy, rough coat, altered gait, priapism, and hunched posture) will be deeply anesthetized by CO<sub>2</sub> and the brain will be removed for analysis.

Inoculated animals are monitored by three "tiers" of individuals and activities. In the first tier, animal caretaker/technicians do a quick visual check of all animals each day, checking for food, water, and general health. The caretakers who perform this daily check are familiar with TSE symptoms, and if any animals seem to be developing symptoms, they are reported to the animal room supervisor. The individuals who regularly perform this daily check are Kris Earll, Willie Paige, and Annette Wise. Other persons who may perform this check if the regular persons are away or unable are Rene'e Kahn and Claudia MacAuley. All persons who do the daily animal health check have been personally trained and approved for this activity by our Senior Animal Technician and Biosafety Officer, Ms. Claudia MacAuley. We maintain a "training checklist" in our laboratory training notebook.



In the second tier, animal caretakers/technicians “score” animals for TSE symptoms once a week. The scoring consists of opening the cage and examining each animal for the specific changes in behavior or appearance. This weekly scoring is begun about one month after inoculation, or when daily checks indicate animals are developing symptoms. The individuals who regularly perform scrapie symptom scoring are Kris Earll, Willie Paige, and Annette Wise. Other persons who may perform the scoring are Rene’e Kahn and Claudia MacAuley. All persons who do symptom scoring are specifically trained and approved for this activity by Ms. MacAuley.

In the third tier, the Director of the lab (myself) or the Senior Animal Technician (Claudia MacAuley) is consulted if there is some question about how to score an animal.

#### *Euthanasia by CO<sub>2</sub> Anoxia*

With respect to the time point of euthanasia, mice in this study will be euthanized : 1) once TSE infection has progressed far enough that it can be conclusively diagnosed by the clinical symptoms AND 2) once the disease has progressed far enough that sufficient PrP<sup>res</sup> protein, or prion protein, has been produced so that the PrP<sup>res</sup> can be reliably detected by the standard Western blot, which is the biochemical assay that we use to confirm TSE diagnosis. With respect to (1), no single symptom is conclusively diagnostic of TSE, but rather a progression of symptoms is required. In this study, the particular symptoms are unknown, since we will be testing strains of TSE that have not been previously tested in a laboratory setting with laboratory animals. We will be formulating criteria for scoring clinical symptoms as the study progresses. With respect to 2), the amount of PrP<sup>res</sup> protein in the brain increases with incubation time of the disease. At pre-symptomatic times, there is not sufficient PrP<sup>res</sup> protein to be detected by the biochemical assay (Western blot). In the case of scrapie in hamsters, enough PrP<sup>res</sup> protein has accumulated by mid-clinical stage (about 5 to 8 days after first symptoms) to give a clear and interpretable Western blot result. With respect to who determines when to perform euthanasia, in this case Dr. Robert Rohwer will decide what set of symptoms constitute a positive clinical diagnosis and will give instructions for the timing of euthanasia. Once this has been established for a given strain of TSE and strain of mouse, the symptom scorers (second tier) will make the decision as to when the animals have reached the clinical stage at which they are to be euthanized. If there is any question, they consult Dr. Rohwer or Ms. MacAuley.

Animals will be placed in a chamber that is sealed with a close-fitting lid, and gaseous CO<sub>2</sub> will be fed from a CO<sub>2</sub> tank through a port into the chamber. Animals will be kept in the chamber until they lose consciousness and cease breathing. The skull is then opened by cutting with dissecting scissors and the brain is removed and frozen for future Western Blot analysis. For further details, see description of procedure immediately below, under “A. Euthanize and harvest tissue”.

### **Additional information for Research Categories Checked on Page 1**

#### **A. Euthanize and harvest tissue**



Mice are euthanized by CO<sub>2</sub> anoxia. Animals are placed one at a time in a polycarbonate chamber with a tight fitting lid and a spigot connected to a regulated CO<sub>2</sub> gas cylinder. CO<sub>2</sub> gas is introduced into the chamber. Each animal is observed for loss of consciousness (about 30 seconds after being placed in chamber) and cessation of all respiratory and other movements (about 2 minutes after being placed in chamber) before being removed from the chamber. Death is confirmed by immediate removal of the brain, or, when the brain is not harvested, by cervical fracture. When the brain is taken, dissection is complete within thirty seconds. When only one or a few animals are being euthanized, the chamber will be free of CO<sub>2</sub> when the animal is first placed in it. When many animals are being euthanized, they will be euthanized individually, and the chamber will be opened between each animal, so that some "airing out" of the chamber occurs, but experimental constraints do not allow complete removal of all CO<sub>2</sub> from the chamber in between individual animals.

#### **L. Biohazardous materials**

##### *Scrapie, Chronic Wasting Disease, and classical Creutzfeldt-Jakob Disease*

Scrapie, CJD, and varCJD are classified as Biosafety Level II agents. Chronic Wasting Disease is unclassified. Scrapie and chronic wasting disease have unknown potential for human disease. Although it is not required to work with these agents in a BL3 laboratory, the principal investigator prefers to work with these agent in a restricted environment, until the potential, or lack thereof, for human disease are more firmly established. Therefore, all mice used for studies with material infected with the above three strains will be maintained in the VA Animal Biosafety Level Three Laboratory, ABSL3.

##### *Chronic Wasting Disease*

Mice transgenic for elk PrP gene will be inoculated with the CWD agent, an unclassified agent with unknown potential for human disease. Nevertheless, the principal investigator prefers to work with this agent in a restricted environment, until its identity and potential, or lack thereof, for human disease are more firmly established. Animals used in these studies will be maintained in the VA Animal Biosafety Level Three Laboratory, ABSL3.

##### *Scrapie, Variant Creutzfeldt-Jakob Disease, and varCJD*

Mice transgenic for the human PrP gene will be inoculated with CJD or varCJD agent, a class II agent with known potential for human disease. Mice transgenic for the sheep PrP gene will be inoculated with scrapie agent, a class II agent with unknown potential for human disease. The principal investigator works with this agent in a restricted environment. All work with scrapie and varCJD-infected animals and scrapie and varCJD -infected materials is done within the BL3 facility, following strict BL3 procedures as described in our Manual of Standard Operating Procedures. Briefly, animals are kept in microisolator cages that are opened only in Biosafety Cabinets. All animal procedures are done within the Biosafety Cabinet, and all laboratory manipulations with BL3 materials are done within the Biosafety Cabinet. Protective clothing including two pairs of gloves is worn at all times. All waste is autoclaved for 1 or 2 hours at 134°C and then incinerated. All animals are incinerated.

#### **O. Specialized Housing.**



Animals used in these studies will be maintained in the VA Animal Biosafety Level 3 Facility in Room BB113. Animals are maintained under microisolators in a room under negative pressure to the ABSL3 facility, which is under negative pressure to the general facility. All caging and cage waste is autoclaved out of the facility before disposal and clean caging is autoclaved back into the facility.

#### **Additional Information on Procedures that produce Category B Pain (Discomfort):**

**Inoculations** - Blood inoculated IC produces a noxious reaction in mice (vocalization, twitching, convulsions) that is generally transient. For this reason, animals are deeply anesthetized with ip-injected pentobarbital (40-60 mg/kg body weight) prior to ic inoculation. After inoculation, animals will be monitored closely during their recovery from anesthesia, and if they show any abnormal behavior (vocalization, twitching, intense scratching of the inoculation site) they will be euthanized immediately by cervical fracture.

**TSE disease** - Animals inoculated with scrapie, CWD, and CJD and <sup>var</sup>CJD will invariably contract TSE disease. These diseases have a long preclinical incubation period during which the animals are unaffected by the infection. The length of this period varies with the dose and route of inoculation, and is unknown for the new strains we will be studying. Animals are euthanized once they have clear clinical symptoms (behavioral changes, inability to feed normally). If allowed to progress, they would continue to lose weight and eventually would no longer be able to feed. TSE infection will begin insidiously and likely have a two or three-week duration. None of the individual symptoms of this disease are pathognomonic; rather it is the progression of disease that defines the disorder. Animals will be segregated and monitored daily if they show abnormal behavior and terminated when they can no longer eat or drink.

It seems likely that clinical TSE causes discomfort in the animals. However there is no evidence that significant pain is experienced in either the animal or the human spongiform diseases. There are currently no palliative measures that can be employed in either humans or animals to relieve the symptoms of the disease

#### **Attachment E- Qualification, Experience, Training**

**Robert G. Rohwer, Ph. D.** – P.I. Dr. Rohwer has twenty-nine years of research experience working with the transmissible spongiform encephalopathy (TSE) agents in hamsters and mice, including both planning and interpreting experiments and performing direct, hands-on animal procedures. He is considered a foremost authority on the titration of TSE infectivity in rodents and developed the limiting dilution titration method that has made the investigation of blood-borne infectivity possible. He has served on the IACUC committee of the University of Maryland for eight years, and is familiar with the regulations for humane and proper treatment of animals.

For this study, Dr. Rohwer will design the experiments and supervise their over-all conduct.



**Renée Kahn, B.S.** – Manager of BL3 Animal Facility. Ms. Kahn has served as manager of the BL3 Animal facility for the Laboratory of Molecular Neurovirology for the past 3 years. Prior to that, she had three years experience as an assistant Manager of animal facilities, and before that fifteen years' experience with laboratory animal research as an animal technician at various levels. She has received training in biohazard management, waste management, Good Laboratory Practice (including certification), and Good Manufacturing Practice. She supervises and participates in the animal husbandry and animal technical work, schedules all animal work, maintains records, and trains Animal Facility staff.

For this study, Ms. Kahn will schedule and coordinate the work of the various technicians and assist where needed.

**Claudia MacAuley, LAT-** BioSafety Officer. Ms. MacAuley is an AALAS Certified Laboratory Animal Technician with twenty-six years of experience in animal technical work. She has worked with Dr. Rohwer in the field of TSE research for the past eleven years, and has been trained by him for work with the spongiform agents. She has completed numerous courses and workshops on biohazard control and biomedical waste management, including an advanced Johns Hopkins course on Safety in the Biological Laboratory. Ms. MacAuley is the senior animal technician for the laboratory, and assists in training all other animal technicians. She is also the Biosafety Officer, and as such monitors the BL3 facility and all procedures to ensure that work is being done safely and properly.

For this study, Ms. MacAuley will carry out technical procedures, including inoculating mice with samples for bioassay and supervising the clinical scoring of animals.

#### **Other Technicians:**

**Kris Earll** – Mr. Earll has four years of experience with laboratory animal care, all of those in the Rohwer lab handling TSE-infected animals. He supervises the animal husbandry of the mouse room in the BL3 Facility and performs animal technical work, including evaluations of disease states, inoculations, bleeding, euthanasias and dissections. He is experienced at monitoring the incubation of new strains of TSE in laboratory rodents.

For this study, Mr. Earll will be in charge of the care of the mice used for bioassay of samples and will perform daily animal checks and weekly disease assessments, and help with inoculations.

**Willie Paige, RLAT-** Mr. Paige is an AALAS-certified Registered Laboratory Animal Technician with twenty years of experience in laboratory animal research, including work with rodents, rabbits, sheep, dogs, cats, snakes, pigs, and non-human primates. Mr. Paige joined the Rohwer laboratory three years ago. He performs animal husbandry and animal technical work, including evaluations of disease states, inoculations, bleeding, euthanasias and dissections.

For this study, Mr. Paige will help with the care of the mice used for bioassay of samples.

**Annette Wise** – Ms. Wise has two years of experience as an animal caretaker and technician with the Humane Society of Baltimore County and a year of experience in the Rohwer laboratory. She has carried out animal husbandry, administered medication, and performed euthanasia for large and small animals. She expects to complete her B.S. in Biology in May, 2004.



For this study, Ms. Wise will assist in the evenings or on weekends with daily checks or weekly disease state assessments, as well as with animal husbandry.



## SUPPORTING DATA:

**Table 1 Titration of natural infections in mice with same-species PrP transgene.**

Titration Method: End-Point Dilution					
Sample:	WHO sCJD I	WHO sCJD II	WHO vCJD	WHO fCJD	Natural Sheep Scrapie
Transgenic	Human	Human	Human	Human	Sheep
Days pi	393	387	386	274	550
Dilution <sup>1</sup>	Infected/inoculated				
10 <sup>-1</sup>	2/2	toxic	0/2	0/5	3/3
10 <sup>-2</sup>	4/4	5/5	0/4	0/5	5/5
10 <sup>-3</sup>	-	5/5	0/4	0/5	5/5
10 <sup>-4</sup>	4/4	5/5	0/5	0/5	4/4
10 <sup>-5</sup>	5/5	0/3	0/4	0/5	4/4
10 <sup>-6</sup>	0/5	0/4	0/5	0/4	0/2
10 <sup>-7</sup>	0/5	0/4	0/4	0/5	0/2
<b>ID/g<sup>1</sup></b>	1.0 x 10 <sup>7</sup>	1.0 x 10 <sup>6</sup>	none	?	1.0 x 10 <sup>7</sup>
Uninoculated control	0/5	0/6	0/2	0/2	0/5
<p>*Does not include animals that died of intercurrent causes, which may or may not have developed scrapie had they lived longer. Five animals were originally inoculated at each dilution.</p> <p><sup>1</sup>Dilution relative to whole brain. ID/g of brain</p>					